THE UNIVERSITY OF QUEENSLAND

AUSTRALIA

Investigating the host and bacterial factors in cystic fibrosis that promote persistence of infection in the lung.

Dr Daniel James Smith
MBChB

A thesis submitted for the degree of Doctor of Philosophy at
The University of Queensland in 2015
School of Medicine

Abstract

Background and aims

Cystic Fibrosis (CF) is a multi-system genetic disease, but it is complications associated with chronic respiratory tract sepsis that most commonly result in premature death. By Adulthood, a chronic poly-microbial lung infection is established, and *Pseudomonas* aeruginosa emerges as the dominant pathogen. Established *P. aeruginosa* infection cannot be eradicated with currently available antibiotics and results in increased morbidity and mortality.

The CF lung is inherently prone to bacterial infections, which are not cleared despite an intense host immune response, suggesting that defects in host innate immune defences may contribute to the pathobiology of CF lung infections.

Iron is essential to critical metabolic pathways in humans and bacteria and is a regulator of phenotypic adaptation in *P. aeruginosa*. Detoxification and sequestration of iron in the human airway is an important anti-microbial defence strategy, which recent studies have suggested may be defective in CF.

The **MAIN AIMS** of this PhD thesis were to examine the factors that may predispose to, and perpetuate, chronic *P. aeruginosa* infection in CF patients including: i) The impact of antibiotic treatment practices in Australian CF centres on rates of *P. aeruginosa* antibiotic resistance, ii) Investigation of bacterial population and host immune related factors that may result in recalcitrant pulmonary infection and modulate the response to anti-microbial therapy, and iii) The impact of disordered systemic and pulmonary iron homeostasis upon the severity of CF lung disease.

Methods

i) **Predicting *P. aeruginosa* antibiotic resistance.**

Data from the Australian clonal *Pseudomonas* in CF study were used to identify trends in *P. aeruginosa* antibiotic resistance in Australian adult and paediatric CF centres and to identify treatment related factors associated with an increased risk of antibiotic resistance.

ii) **Response of the CF lung microbiome to antibiotic therapy**

Bacterial DNA was extracted from expectorated sputa of subjects with CF. The 16S rRNA gene was amplified and sequenced by bacterial tag-encoded FLX amplicon pyrosequencing to determine the composition and relative abundance of bacterial genera. Changes in microbial community composition in response to intravenous antibiotic therapy were determined.

iii) **Adaptive immune responses to infection in the CF**

Multi-colour flow-cytometry was used to phenotype peripheral blood lymphocytes in CF and healthy subjects. Phenotypic profiles were correlated with indices of disease severity and infection.

iv) **Biometal composition of the CF airway**

Inductively-coupled plasma mass-spectrometry was used to detect iron and the presence of other bio-active metals in sputum samples from CF and healthy control subjects. Airway biometal composition was correlated to indices of disease severity, airway inflammation and cellular injury.

v) **Haemochromatosis modifier gene effects in CF**

Haemochromatosis (*HFE*) genotyping was performed on 163 adults, attending a single CF centre. The impact of *HFE* gene mutations upon disease phenotype and systemic iron homeostasis were examined.

Results

Rates of multi-antibiotic resistant *P. aeruginosa* (MARPA) were high in Australian CF subjects infected with *P. aeruginosa*. In large paediatric centres, increased antibiotic usage was associated with MARPA. In adult and paediatric centres, the centre of care predicted for MARPA. Infection with a major shared Australian *P. aeruginosa* strain correlated with increased treatment burden and MARPA.

Culture independent analysis of the sputum microbiome of 23 adult CF subjects infected with *P. aeruginosa*, demonstrated an increased relative abundance of Pseudomonas was associated with overall reduced microbial diversity. Administration of intravenous antibiotics resulted in an early reduction in the abundance of Pseudomonas at a genus level, however, this was not sustained beyond the first week of antibiotic treatment.

In comparison to healthy controls, peripheral blood from CF subjects contained a reduced proportion of mucosal associated invariant T-lymphocytes (MAIT cells). In CF, reduced MAIT cell

concentration was correlated with *P. aeruginosa* infection, increased disease severity and pulmonary exacerbations.

Sputum of CF subjects contained increased concentrations of iron, magnesium, calcium, and zinc. Metal concentrations correlated with airway inflammation and cellular injury. CF subjects with severe disease had elevated sputum molybdenum concentrations.

CF subjects with a C282Y haemochromatosis mutation experienced accelerated lung function decline and had an increased risk of CF related diabetes and gastro-intestinal complications.

Conclusion

This thesis describes wide variations in the rates of MARPA and treatment practices between CF centres across Australia and suggests the need to develop national treatment guidelines in order to maximise pulmonary outcomes and minimise treatment related complications.

The CF airway bacterial microbiome of adults with established disease is complex and relatively resistant to intravenous antibiotic treatment. Sputum of CF subjects contains increased concentrations of bio-active metals which correlate with risk of *P. aeruginosa* infection, airway inflammation and cellular damage.

MAIT cells concentrations were reduced in the blood of subjects with CF and associated with important markers of pulmonary disease severity, suggesting their concentrations may represent a systemic biomarker of CF lung disease.

Finally the association of the C282Y haemochromatosis gene mutation with increased lung disease severity, risk of diabetes and gastro-intestinal complication in CF subjects, suggest *HFE* may be a modifying gene in cystic fibrosis, which offers the opportunity to therapeutically intervene.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

I acknowledge that an electronic copy of my thesis must be lodged with the University Library and, subject to the policy and procedures of The University of Queensland, the thesis be made available for research and study in accordance with the Copyright Act 1968 unless a period of embargo has been approved by the Dean of the Graduate School.

I acknowledge that copyright of all material contained in my thesis resides with the copyright holder(s) of that material. Where appropriate I have obtained copyright permission from the copyright holder to reproduce material in this thesis.

Publications during candidature

Peer reviewed publications

i) **Smith DJ**, Hill GR, Bell SC, Reid DW. Reduced mucosal associated invariant T-cells are associated with increased disease severity and *Pseudomonas aeruginosa* infection in cystic fibrosis. Plos One 2014;9(10)e109891.

ii) **Smith DJ**, Badrick AC, Zakrzewski M, Krause L, Bell SC, Anderson GJ, Reid DW. Pyrosequencing reveals transient CF lung microbiome changes with intravenous antibiotics. European Respiratory Journal 2014;44(4):922-30.

iii) **Smith DJ**, Anderson GJ, Bell SC, Reid DW. Elevated metal concentrations in the CF airway correlate with cellular injury and disease severity. Journal of Cystic Fibrosis 2014;13(3):289-95.

iv) Houston N, Stewart N, **Smith DJ**, Bell SC, Champion A, Reid DW. Sputum neutrophils in cystic fibrosis patients display a reduced respiratory burst. Journal of Cystic Fibrosis 2013;12(4):352-362.

v) **Smith DJ**, Lamont IL, Anderson GJ, Reid DW. Targeting iron uptake to control *Pseudomonas aeruginosa* infections in cystic fibrosis. European Respiratory Journal 2013;42(6):1723-1736

vi) Wood ME, **Smith DJ**, Reid DW, Masel PJ, France MW, Bell SC. Ivacaftor in severe cystic fibrosis lung disease and a G551D mutation. Respirology Case Reports 2013;1(2):52-54

vii) Kidd TJ, Ramsay KA, Marks GB, Bye PT, Elkins MR, Robinson PJ, Rose BR, Wilson JW, Grimwood K, Bell SC and the **ACPinCF Investigator Group***. Shared *Pseudomonas aeruginosa* genotypes are common in Australian cystic fibrosis centres. European Respiratory Journal 2013;41(5):1091-1100.

viii) **Smith DJ**, Reid DW, Bell SC. Treatment of pulmonary exacerbations in cystic fibrosis. Therapy 2011;8(6):623-643

* I am a named member of the ACPinCF Investigator Group

Letters

i) **Smith DJ**, Anderson GJ, Lamont IL, Masel P, Bell SC, Reid DW. Accurate assessment of systemic iron status in cystic fibrosis will avoid the hazards of inappropriate iron supplementation: Letter to the Editor. Journal of Cystic Fibrosis 2013;12:303-304

Manuscripts currently under consideration

i) **Smith DJ**, Ramsay KA, Yerkovich ST, Reid DW, Wainwright C, Grimwood K, Bell SC, Kidd TJ. *Pseudomonas aeruginosa* antibiotic resistance in Australian cystic fibrosis centres. Under review with Respirology: Official Journal of the Asian Pacific Society of Respirology

ii) **Smith DJ**, Klein K, Wainwright C, Anderson GJ, Bell SC, Reid DW. Mutations in the *HFE* gene are associated with increased lung disease severity in cystic fibrosis. Under review with the journal Plos One

Presentations at international scientific meetings

i) **Smith DJ**, Hill GR, Bell SC, Reid DW. Reduced mucosal associated invariant T-cells are associated with increased disease severity and *Pseudomonas aeruginosa* infection in cystic fibrosis. Thoracic Society of Australia and New Zealand, Gold Coast 2015

ii) **Smith DJ**, Bell SC, Anderson GJ, Reid DW. *HFE* 282Y mutations are associated with severity of lung disease and rate of lung function decline in cystic fibrosis. Thoracic Society of Australia and New Zealand ASM, Adelaide 2014

iii) **Smith DJ**, Anderson GJ, Bell SC, Reid DW. Increased bio-active metals in the sputum of patients with cystic fibrosis and association with disease severity. Australasian CF Conference ASM, Auckland 2013

iv) **Smith DJ**, Smith A, Anderson GJ, Krause L, Zakrzewski M, Bell SC, Reid DW. Perturbation of sputum bacterial community composition by intravenous antibiotics in cystic fibrosis is short-lived. Australasian CF Conference ASM, Auckland 2013 and Thoracic Society of Australia and New Zealand ASM, Darwin 2013

v) **Smith DJ**, Watts RE, Anderson GJ, Bell SC, Reid DW. Iron chelation ameliorates iron induced neutrophil oxidative stress and restores oxidative burst capacity. Australasian CF Conference ASM, Auckland 2013

vi) **Smith DJ**, Badrick A, Anderson GJ, Bell SC, Reid DW. Iron supplementation and defective iron homeostasis are associated with worse lung function in Cystic Fibrosis. Thoracic Society of Australia and New Zealand ASM, Canberra 2012.

vii) **Smith DJ**, Kidd T, Ramsay K, Wainwright C, Grimwood K, Bell SC. *Pseudomonas aeruginosa* antibiotic resistance: Comparison between Australian Cystic Fibrosis (CF) Centres. Thoracic Society of Australia and New Zealand ASM, Canberra 2012.

viii) **Smith DJ**, Reid DW, Tai A, Masel P, Slaughter R, Moore V, Bell SC. Superior vena cava syndrome due to total implantable venous access devices in cystic fibrosis. Thoracic Society of Australia and New Zealand ASM, Perth 2011.

ix) **Smith DJ**, Houston N, Reid, DW. Neutrophils within the cystic fibrosis airway display a reduced respiratory burst. ComBio ASM, Cairns 2011.

x) **Smith DJ**, Kidd TJ, Ramsay K, Grimwood K, Wainwright C, Bell SC. *Pseudomonas aeruginosa* antibiotic resistance: comparison between Australian paediatric and adult cystic fibrosis centres. Australasian CF Conference ASM, Melbourne 2011.

xi) **Smith DJ**, Houston N, Stewart N, Champion A, Reid DW. Neutrophil viability and oxidative burst capacity within the Cystic Fibrosis airway. Australasian CF Conference ASM, Melbourne 2011.

Publications included in this thesis

This thesis includes excerpts from two review manuscripts and three original research manuscripts that have been published in peer-reviewed international journals, and two original research manuscripts that are under consideration for publication in peer-reviewed international journals.

Chapter 1 contains excerpts from "Targeting iron uptake to control *Pseudomonas aeruginosa* infections in cystic fibrosis" (Smith, DJ, et al. *European Respiratory Journal* 2013;42(6):352-362) and "Treatment of pulmonary exacerbations in cystic fibrosis" (Smith, DJ, et al. *Therapy* 2011;8(6):623-643). These publications were contributed to by several authors. Specific contributions to writing and editing these reviews by each of the authors are listed below.

Targeting iron uptake to control *Pseudomonas aeruginosa* infections in cystic fibrosis

Name	Statement of Contribution	
Daniel J Smith	Literature review	100%
	Wrote, formatted and edited the paper	70%
Iain L Lamont	Wrote and edited the paper	5%
Gregory J Anderson	Principal supervisor, wrote and edited the paper	10%
David W Reid	Principal supervisor; wrote and edited the paper	15%

Treatment of pulmonary exacerbations in cystic fibrosis

Name	**Contribution**	
Daniel J Smith	Literature review	100%
	Wrote, formatted and edited the paper	70%
David W Reid	Principal supervisor; wrote and edited the paper	10%
Scott C Bell	Associate supervisor; wrote and edited the paper	20%

The manuscript appearing as chapter 2: "*Pseudomonas aeruginosa* antibiotic resistance in Australian cystic fibrosis centres", is under review with *Respirology: Official Journal of the Asian Pacific Society of Respirology* and was contributed to by several authors. Specific contributions to writing and editing of this manuscript by each of the authors are listed below.

Name	Contribution	
Daniel J Smith	Study conception and design	50%
	Acquisition of data	30%
	Analysis and interpretation of the data	30%
	Wrote, formatted and edited the paper	40%
Kay A Ramsay	Acquisition of data	20%
	Wrote, formatted and edited the paper.	5%
Stephanie T Yerkovich	Analysis and interpretation of data	20%
	Wrote, formatted and edited the paper	5%
David W Reid	Principal supervisor; acquisition of data	10%
	Analysis and interpretation of the data	10%
	Wrote, formatted and edited the paper	5%
Claire Wainwright	Study conception and design	10%
	Acquisition of data	10%
	Analysis and interpretation of the data	10%
	Wrote, formatted and edited the paper	5%
Keith Grimwood	Study conception and design	10%
	Analysis and interpretation of the data	10%
	Wrote, formatted and edited the paper	10%
Scott C Bell	Associate supervisor; study conception and design	10%
	Acquisition of data	10%
	Analysis and interpretation of the data	10%
	Wrote, formatted and edited the paper	15%
Timothy J Kidd	Study conception and design	20%
	Acquisition of data	20%
	Analysis and interpretation of the data	10%
	Wrote, formatted and edited the paper	15%

The manuscript appearing as chapter 3: "Pyrosequencing reveals transient CF lung microbiome changes with intravenous antibiotics" (Smith, D.J., et al. *European Respiratory Journal* 2014;44(4):922-30) was contributed to by several authors. Specific contributions to writing and editing of this manuscript by each of the authors are listed below.

Name	Contribution	
Daniel J Smith	Study conception and design	50%
	Acquisition of data	40%
	Analysis and interpretation of the data	30%
	Wrote, formatted and edited the paper	40%
Alison C Smith	Acquisition of data	40%
	Analysis and interpretation of the data	10%
	Wrote, formatted and edited the paper	15%
Martha Zakrzewski	Analysis and interpretation of the data	30%
	Wrote, formatted and edited the paper	2.5%
Lutz Krause	Analysis and interpretation of the data	10%
	Wrote, formatted and edited the paper	2.5%
Scott C Bell	Associate supervisor, wrote, formatted and edited the paper	5%
Gregory J Anderson	Principal supervisor, wrote, formatted and edited the paper	5%
David W Reid	Principal supervisor; study conception and design	50%
	Acquisition of data	20%
	Analysis and interpretation of the data	20%
	Wrote, formatted and edited the paper	30%

The manuscript appearing as chapter 4: “Mucosal associated invariant T-cells predict lung disease severity and *Pseudomonas aeruginosa* infection in cystic fibrosis.” (Smith, D.J., et al. Plos One 2014;9(10):e109891) was contributed to by several authors. Specific contributions to writing and editing of this manuscript by each of the authors are listed below.

Name	Contribution	
Daniel J Smith	Study conception and design	70%
	Acquisition of data	80%
	Analysis and interpretation of the data	70%
	Wrote, formatted and edited the paper.	70%
Geoffrey Hill	Study conception and design	10%
	Analysis and interpretation of the data	10%
	Wrote, formatted and edited the paper	5%
Scott C Bell	Associate supervisor; acquisition of data	10%
	Wrote, formatted and edited the paper.	5%
David W Reid	Principal supervisor; Study conception and design	20%
	Acquisition of data	10%
	Analysis and interpretation of the data	20%
	Wrote, formatted and edited the paper	20%

The manuscript appearing as chapter 5: “Elevated metal concentrations in the CF airway correlate with cellular injury and disease severity.” (Smith, D.J., et al. *Journal of Cystic Fibrosis* 2014;13(3):289-295) was contributed to by several authors. Specific contributions to writing and editing of this manuscript by each of the authors are listed below.

Name	Contribution	
Daniel J Smith	Study conception and design	60%
	Acquisition of data	80%
	Analysis and interpretation of the data	70%
	Wrote, formatted and edited the paper.	70%
Gregory J Anderson	Principal supervisor, study conception and design	10%
	Analysis and interpretation of the data	10%
	Wrote, formatted and edited the paper	5%
Scott C Bell	Associate supervisor; acquisition of data	10%
	Wrote, formatted and edited the paper.	5%
David W Reid	Principal supervisor; Study conception and design	30%
	Acquisition of data	10%
	Analysis and interpretation of the data	20%
	Wrote, formatted and edited the paper	20%

The manuscript appearing as chapter 6: "Mutations in the *HFE* gene are associated with increased lung disease severity in cystic fibrosis." is under review with the journal *Plos One* and was contributed to by several authors. Specific contributions to writing and editing of this manuscript by each of the authors are listed below.

Name	Contribution	
Daniel J Smith	Study conception and design	60%
	Acquisition of data	70%
	Analysis and interpretation of the data	60%
	Wrote, formatted and edited the paper	60%
Kerenaftali Klein	Analysis and interpretation of the data	20%
Claire Wainwright	Wrote, formatted and edited the paper	7.5%
Gregory J Anderson	Principal supervisor, analysis and interpretation of the data	10%
	Wrote, formatted and edited the paper	7.5%
Scott C Bell	Associate supervisor; acquisition of data	10%
	Wrote, formatted and edited the paper	7.5%
David W Reid	Principal supervisor; study conception and design	40%
	Acquisition of data	20%
	Analysis and interpretation of the data	10%
	Wrote, formatted and edited the paper	17.5%

Contributions by others to the thesis

The overall research direction, study design, execution of experiments and writing of the manuscripts pertaining to these experiments was primarily my responsibility as a PhD candidate. Any contribution by co-authors to the research manuscripts which contribute to this thesis are listed in the previous section; “Publications included in this thesis”. Editing and proof reading of this thesis was performed by Associate Professor David Reid, Professor Gregory Anderson and Professor Scott Bell.

Statement of parts of the thesis submitted to qualify for the award of another degree

None

Acknowledgments.

To bring this PhD thesis submission to completion, I am indebted to countless people for their advice and support.

First and foremost, I would like to thank my advisory team. To my principal advisor Associate Professor David Reid, for the encouragement to take on this research project, expert advice throughout, patience in the editing and re-editing the accompanying manuscripts and for remaining a calm sounding board, who patiently helped me work through the complications and frustrations that can accompany such a project. Professor Scott Bell, for his generosity of time, sharing his wealth of experience in production in world class research and clinical CF care and for constantly challenging me to produce high quality work. Professor Greg Anderson, for his advice with regards to understanding iron metabolism in health and disease and study design.

To Dr Stephanie Yerkovich, Ned Houston and Maxine Tan of the Lung Transplant and Pulmonary Hypertension Laboratory, the Prince Charles Hospital and the post-doctoral staff of the Iron Metabolism, Lung Infection and Inflammation, Human immunity and Bone Marrow Transplantation Laboratories of QIMR Berghofer Medical Research Institute for teaching me the research techniques used throughout this thesis and for their assistance with study design. To Michelle Wood and Kenneth Sinclair, for their day to day friendship, which was invaluable in maintaining sanity, through the many long hours dedicated to the completion of this thesis.

A special thanks is given to all the patients of the Adult Cystic Fibrosis Centre of the Prince Charles Hospital, for their willingness to participate in the studies included within this thesis.

The financial assistance of the National Health and Medical Research Council of Australia and The Prince Charles Hospital is gratefully acknowledged.

Finally, special thanks should be given to my wife, Katrina, who offered constant love, support, tolerance and understanding throughout, without which, completion of this thesis would not have been possible.

Keywords.

Cystic fibrosis, *Pseudomonas aeruginosa*, antimicrobial resistance, microbiome, immunity, iron, haemochromatosis.

Australian and New Zealand Standard Research Classifications (ANZSRC).

110203 Respiratory Diseases	60%
110311 Medical Genetics (excl. Cancer Genetics)	10%
110801 Medical Bacteriology	30%

Fields of Research (FoR) Classifictaion

FoR Code:	1102 Cardiorespiratory Medicine and Haematology	60%
FoR Code:	1108 Medical Microbiology	20%
FoR Code:	1107 Immunology	20%

Table of Contents

List of Tables:

List of Figures:

List of Abbreviations

ABC:	ATB-Binding Cassette
ACD:	Anaemia of chronic disease
ACFDR:	Australian CF data registry
ACPinCF:	Australian clonal *Pseudomonas aeruginosa* in Cystic Fibrosis
AEC:	Airway epithelial cell
AM:	Alveolar macrophages
ARDS:	Acute respiratory distress syndrome
ASL:	Airway surface liquid
ATP:	Adenosine triphosphate
AUST:	Australian Shared *Pseudomonas aeruginosa* strain
BALF:	Bronchoalveolar lavage fluid
BCC:	*Burkholderia cepacia* complex
BD:	Twice daily
BMI:	Body mass index
BMP:	Bone morphogenic protein
Ca:	Clacium
CCS:	Canonical correlation analysis
CD:	Cluster of differentiation
CDS:	Continuous Dichotomous Susceptibility
CF:	Cystic Fibrosis
CLSI:	Clinical and Laboratory Standards Institute
CFLD:	CF liver disease
CFRD:	CF related diabetes
CFTR:	Cystic Fibrosis Trans-membrane conductance Regulator
Cl^-:	Chloride
CRP:	C-reactive protein
Cu:	Copper
DIOS:	Distal intestinal obstruction syndrome
DNA:	Deoxyribonucleic acid
DMSO:	Dimethyl sulfoxide
DMT:	Divalent metal transporter
DYCTB:	Duodenal cytochrome-b
ECM:	Extra-cellular matrix

eDNA: Extracellular DNA

ENaC: Epithelial sodium channel

ERIC-PCR: Enterobacterial repetitive intergenic consensus PCR

EUCAST: European committee on antimicrobial sensitivity testing

FACS: Flow cytometry

Fe: Iron

FEV_1: Forced expiratory volume in one second

Fur: Ferric uptake regulator

FVC: Forced vital capacity

G-CSF: Granulocyte colony stimulating factor

GWAS: Genome wide association study

HBECs: Human bronchial epithelial cells

HCL: Hydrochloric acid

HCV: Hepatitis C virus

HFE: Haemochromatosis gene

HH: Hereditary haemochromatosis

HiFCS: Heat-inactivated fecal calf serum

HIV: Human immunodeficiency virus

H_2O_2: Hydrogen peroxide

HOCl: Hypochloric acid

HPLC-MS: High-performance liquid chromatography mass spectrometry

HTS: Hypertonic saline

ICP-MS: Inductively coupled plasma mass-spectrometry

IFN: Interferon

IL: Interleukin

iNKT: Invariant natural killer T cell

IRE: Iron responsive element

IV: Intravenous

LDH: Lactate dehydrogenase

LPS: Lipopolysaccharide

MAIT: Mucosal associated invariant T cell

MARPA: Multi-antibiotic resistance

MBL: Mannose binding lectin

Mg: Magnesium

MHC: Major histocompatibility complex

MI: Meconium ileus

MIC: Minimum inhibitory concentration

Mn: Mananese

Mo: Molybdenum

MR1: MHC related protein 1

mRNA: Messenger RNA

MRSA: Methicillin resistant Staphylococcus aureus

MSSA: Methicillin sensitive Staphylococcus aureus

MTB: *Mycobacterium tuberculosis*

Na^+: Sodium

NCFB: Non-CF bronchiectasis

NFκβ: Nuclear factor kappa beta

Ni: Nickel

NK: Natural killer

NRAMP: Natural resistance-associated macrophage protein

OTU: Operational taxonomic unit

Pb: Lead

PBMC: Peripheral blood mononuclear cells

PCoA: Primary coordinates analysis

PCR: Polymerase chain reaction

PE: Pulmonary exacerbation

PERT: Pancreatic enzyme replacement therapy

PM_{10}: Particulate matter 10 micrometres

PMA: Propidium monoazide

PMN: Polymorphonuclear cells

QiD: Four times daily

QS: Quorum sensing

RCT: Randomised controlled trial

RNA: Ribosomal nucleic acid

ROS: Reactive oxygen species

(r)RNA: Ribosomal RNA

rDNA: Ribosomal DNA

RT-qPCR: Real time quantitative PCR

SD: Standard deviation

SDS: Sodium dodecyl sulphate

SNP:	Single nucleotide polymorphism
SOD:	Superoxide dismutase
sRNA:	Small RNA
STfR:	Soluble transferrin receptors
TCC:	Total inflammatory cell count
TCR:	T-cell receptor
TDS:	Three times daily
TfR:	Transferrin receptor
Th:	T-helper cell
TLR:	Toll like receptor
TNF:	Tumour necrosis factor
TP:	Time-point
Treg:	Regulatory T-cell
TS:	Transferrin saturation
UK:	United Kingdom
USA:	United States of America
VAP:	Ventilated associated pneumonia
WCC:	White cell count
Zn:	Zinc

Chapter 1 Review of the Literature

1.1 Cystic Fibrosis

1.1.1 Introduction

Cystic fibrosis (CF) is the most common autosomal recessive, inherited, life-limiting condition to affect Caucasian populations, with a birth incidence of approximately 1 in 3200(1). Disease manifestations include gastro-intestinal, endocrine and male reproductive dysfunction. However, it is the complications associated with bronchiectasis and chronic respiratory tract infections that result in the greatest degree of morbidity, and early mortality associated with CF.

In recent years advances in CF care, including pancreatic enzyme replacement therapy (PERT), nutritional supplementation, early and aggressive treatment of infection, and the development of specialist centre care, have led to substantial improvements in life expectancy(2). However, the majority of patients still die from complications associated with chronic respiratory sepsis before their fifth decade of life(1, 3). Equally improvements in survival have come at the cost of treatment related morbidity, including the emergence of multidrug resistant infections, complications of indwelling intravenous lines, and allergic and toxic effects of antibiotics in some patients(4-6).

1.1.2 Cellular Defect

CF results from mutation of both allelic copies of the cystic fibrosis trans-membrane conductance regulator (*CFTR*) gene located on long arm of chromosome 7(7). The *CFTR* gene encodes the 1480 amino acid CFTR protein, which functions as an adenosine triphosphate (ATP)-Binding cassette (ABC) transporter ion channel (8). CFTR is expressed in high concentrations on the apical surface of epithelial cells lining the lung, gastro-intestinal tract, and sweat glands. At these sites CFTR primarily functions as a chloride (Cl^-) ion channel. In addition CFTR co-localises with, and regulates the function of the epithelial sodium (Na^+) channel (ENaC)(9).

At the primarily secretory epithelial surfaces of the airways and gastro-intestinal tract, CFTR inhibits proteolysis of ENAC extracellular domains, thereby inhibiting its function(10). In tandem, CFTR activation, and inhibition of ENAC limits Na^+ and Cl^- reabsorption across the apical cell membrane of epithelial cells, thereby promoting diffusion of water down the osmotic gradient to the luminal surface. In contrast, in the primarily absorptive epithelial cells lining sweat glands CFTR

displays an opposite, positive regulatory effect on ENAC function(11). Consequently, defects in the production or function of CFTR result in dehydration of airway surface liquid (ASL) and inspissation of gastro-intestinal secretions, and excessive loss of salt and water in sweat.

Following initial transcription CFTR undergoes extensive post-transcriptional modification and regulation on the endoplasmic reticulum and Golgi apparatus before being transported aboard clathrin coated vesicles to the apical cell membrane(12). Nearly 2000 different mutations within the *CFTR* gene have been described(13), however, these can be broadly divided into six functional classes, dependent upon the effect they have upon the transcription and post-transcription modification of CFTR (Table 1-1)(14, 15).

Table 1-1 Classification of *CFTR* gene mutations.

Class	Functional effect of mutation	Common phenotype causing mutations
I	Defective protein production	G542X, R553X, W1282X
II	Defective protein processing	DF508del, N1303K
III	Defective protein regulation	G551D, R560T
IV	Defective protein conductance	R117H, R334W, G85E
V	Reduced amount of functioning protein	A455E, 2789+5G→A
VI	Increased turnover of functional protein	120del23, N287Y

1.1.3 Modifier Genes.

In general, class I-III *CFTR* mutations are associated with a more severe "classical" disease phenotype, with class IV-VI being associated with "atypical" milder disease, and late diagnosis(15-17). However, disease phenotype is variable between individuals with similar CF genotypes. In part, this variability may reflect different environmental exposures, however, the influence of modifier genes has also been postulated(18). Gene modifiers may alter target gene function or modify phenotype at the molecular, cellular, systemic or organismal level(19).

A combination of candidate gene and gene wide association studies (GWAS) have been used to identify genes which may influence CF disease phenotype(18, 20, 21). To date, genes which influence the function of immune mediators (including mannose binding lectin, transforming

growth factor β, interferon-related developmental regulator 1 and interleukin-1), anti-oxidants (e.g. glutathione pathways), ion transporters (e.g. solute carrier family genes) and pancreatic β-cell function (*transcription factor 7-like 2 gene*) have been implicated in modifying CF phenotype(18, 20, 22-24).

1.1.4 Clinical manifestations.

CF is a multisystem disease, however the greatest burden of disease falls upon the respiratory, endocrine, gastro-intestinal, hepato-biliary, and male reproductive systems. Subjects with the most severe phenotype typically present in the neonatal period with meconium ileus (MI) (impaction of inspissated intestinal secretion resulting in complete bowel obstruction), failure to thrive, or are diagnosed on the basis of an elevated "heal-prick" trypsinogen concentrations in countries such as Australia where new born screening is employed(2).

Patients with a "classic" disease phenotype develop exocrine pancreatic insufficiency in the early neonatal period, and require lifelong PERT to prevent malabsorption. Over 30% of patients will develop diabetes by the third decade of life, with other common long term complications including chronic liver and bone disease(1, 2). However, the greatest burden of disease for the majority of subjects with CF is due to chronic pulmonary infection. To limit morbidity, sufferers are required to perform daily physiotherapy and use inhaled therapies to clear tenacious airway secretions, take regular course of antibiotics, and undergo recurrent hospitalisation for the treatment of infective pulmonary exacerbations.

1.2 Pulmonary immune defences in CF

The human lung employs physical, humoral, and cellular defence strategies to prevent chronic bacterial infection. It has been proposed that inherent defects in immune responses exist in CF and that these contribute towards the persistence of infection(25). Furthermore, it has been demonstrated that the previously considered "pristine", non-infected neonatal CF lung contains increased concentrations of immune cells, a finding which has been used to support the hypothesis that pulmonary inflammation may precede infection, although this remains a matter of debate(26-28).

1.2.1 Airway surface liquid

Airway surface liquid (ASL) contains high concentrations of mucin, a large heterogeneous glycoprotein with variable carbohydrate side chains which are capable of trapping inhaled bacteria

(29). In healthy airways, inhaled bacteria adhere to mucins and are rapidly cleared by the muco-ciliary escalator. In CF, increased Na^+ and water reabsorption dehydrates ASL making it "sticky" and slow moving. Additionally, serine proteases released by invading pathogens degrade mucin and further compromise the rheological properties of ASL(30). Consequently, inhaled bacteria have the opportunity to multiply and establish infection before they are cleared. Furthermore, CF ASL is deficient in a number of humoral, anti-bacterial mediators, including the iron binding protein lactoferrin, hypothiocyanite, and surfactant protein A(31-33).

1.2.2 Airway epithelial cells

Airway epithelial cells (AECs) represent the primary interface between inhaled pathogens and the immune system and are vital to the coordination of normal innate and adaptive immune responses.

Abnormal signalling and anti-inflammatory responses by CF AECs may promote sustained inflammation within the CF lung(25). *In vitro,* CF AECs show increased expression of genes involved in pro-inflammatory signalling pathways, in the absence of infective stimuli(34). Differences in CF AECs cytokine responses include increased production of the neutrophil chemoattractant interleukin (IL)-8 and the pro-inflammatory nuclear factor kappa-beta (NFkB), and decreased production of anti-inflammatory IL-10 in response to *P. aeruginosa* LPS stimulation(34). CF AECs also display deficient production of the anti-oxidant glutathione and exaggerated production of pro-inflammatory prostaglandins and cyclo-oxygenase(35, 36).

The mechanism by which defective CFTR functioning results in abnormal AEC responses is unknown, although recent work has proposed that the loss of normal CFTR inhibition of intracellular calcium flux may influence gene expression profiles(37).

1.2.3 Polymorphonuclear cells

Polymorphonuclear cells (PMNs, neutrophils) are the primary cell of the innate immune response, and dominate airway inflammation in CF(38). Airway neutrophil numbers correlate with both the severity and progression of CF lung disease.

In "healthy" people, the typical response to bacterial infection is rapid recruitment of neutrophils to the site of infection, driven by local production of chemo-attractants. At the site of infection,

bacteria are phagocytosed and killed by activated neutrophils, which then trigger self-death mechanisms (apoptosis) and are cleared by airway macrophages(39).

Neutrophil intracellular killing occurs within the phagolysosome and is reliant on the "oxidative burst" in which reactive oxygen species (ROS) are generated in a step-wise manner(38). Although each of these oxygen species is toxic in its own right, hypochloric acid (HOCl) and hydrogen peroxide (H_2O_2) are considered the most important for bacterial killing(40).

Neutrophils from people with CF demonstrate distinct gene expression profiles which suggest inherent abnormalities in neutrophil function may exist(41). CFTR expression in neutrophils co-localises with intra-cellular phagosomes and *in vitro CFTR* mutant neutrophils exhibit reduced bacterial killing and chlorination of bacterial proteins(40). It is postulated that loss of CFTR function may result in reduced chlorination of phagolysosome and reduced production of HOCl, which culminate in ineffective bacterial killing(40). Furthermore, CF neutrophils demonstrate abnormal expression of toll like receptors (TLR's), inflammatory cytokines (including IL-8) and reduced production of the anti-inflammatory protein annexin(25, 41, 42).

1.2.4 Alveolar Macrophages

Alveolar macrophages (AM) are involved in direct microbial killing and the removal of apoptotic PMNs (efferocytosis), and modulate the adaptive immune response through antigen presentation.
As for neutrophils, CFTR is expressed within the lysosomes of alveolar macrophages(43), and CF AM display defective bacterial killing capabilities(25). Initial studies in a murine CF model suggested loss of CFTR function may lead to defective acidification of intracellular lysosomes(43). However, the results of this study and the experimental techniques employed have been refuted in subsequent studies(44). To date, an alternate mechanism has not been proposed.

There are few studies of the efficacy of efferocytosis and antigen presentation by CF AM. A single study has suggested that efferocytosis is not impaired in CF AM(45). While *ex vivo* examination of alveolar monocytes (macrophages and dendritic cells) from explanted CF lungs suggest these cells have an impaired ability to present antigen, despite circulating monocytes from the same patient having normal antigen presenting capabilities. This finding suggests an inducible monocyte defect caused by local factors in the CF lung as opposed to an inherent cellular defect(46).

1.2.5 Lymphocytes.

At the level of the airway lumen, the cellular immune response is dominated by activated neutrophils. In contrast, airway epithelial biopsies demonstrate a profound T lymphocyte (T-cell) infiltrate from an early age(47). Furthermore sub-mucosal lymphocyte concentrations correlate with regions of high tissue destruction and *P. aeruginosa* density, suggesting an important role in disease severity(47, 48).

T-cells are instrumental in orchestrating adaptive immune responses. Historically T-cell responses was dichotomised to either, T helper (Th)-1 predominant, in which interferon (IFN)-γ production resulted in macrophage activation and a cellular immune response, or Th-2 predominant, in which IL-4 production resulted in a humoral response and activation of mast cells. This paradigm has since been superseded by a greater understanding of the complexity of T-cell responses and identification of other important T-cell populations. Particular attention in the setting of chronic pulmonary infection has been given to Th-17 and regulatory T-cells (T_{reg}-cells, and in more recent times, "innate" T-cells.

Initial studies of peripheral, adaptive immune responses in CF focused on the dichotomy of T-helper (Th)-1 and Th-2 responses(49). These early studies suggested a skew towards a Th-2 in the majority of CF subjects infected by *P. aeruginosa*, which resulted in increased pulmonary inflammation and disease progression. However, in a minority of patient a predominant Th-1 responses was associated with a more indolent course(49-51).

Th-17 cells represent an IL-17 producing sub-population of T-cells, which develop from naïve T-cells in response to IL-23 stimulation(52). IL-17 stimulates granulopoiesis and neutrophil recruitment, as well as mediating tissues destruction, by regulating the production of matrix metalloproteinases (MMPs)(53). Animal models suggest CFTR deficient, naïve T-cells have an intrinsic propensity to differentiate towards a Th-17 lineage(54). Clinically, sub-mucosal biopsies taken from the airways of children with CF demonstrate a high concentration of IL-17 positive T-cells(55). Furthermore, increased concentrations of IL-17 and other Th-17 related cytokines in bronchioalveolar lavage fluid (BALF) correlate with disease severity, pulmonary exacerbations and acquisition of *P. aeruginosa*(56). The strong relationships between Th-17 mediators and airway inflammation have resulted in the postulate that CF lung disease may be a Th-17 mediated(57, 58).

Treg cells, which produce predominantly anti-inflammatory cytokines (including IL-10, and TGF-β) are believed to play a crucial role in moderating T-cell responses to infections and preventing autoimmunity(59). In the lung, an imbalance in Th17/Treg numbers in favour of a pro-inflammatory (Th17) response has been linked with acute exacerbations of chronic obstructive pulmonary disease and reduced in Treg cell number and activity has is associated with increased risk of atopic asthma(60, 61). Conversely, excessive Treg cell responses have been implicated in impeding the clearance of Mycobacterial infections and adequate response to severe sepsis(62, 63).

To date, studies into the role of Treg cells in CF related lung disease are limited. Anil and Singh demonstrated that children with CF have reduced numbers of peripheral blood Treg cells when compared to healthy controls and that in these children Treg cell numbers were inversely correlated with lung function(64). These finding were replicated in a more recent study by Hector *et al*, which also included data from adult patients and additionally demonstrated that chronic infection with *P. aeruginosa* caused a further reduction in peripheral blood Treg cell number(65). The authors of this later study went on to demonstrate that Treg cell numbers were lower in the bronchoalveolar lavage fluid of patients with CF compared to patients with non-CF bronchiectasis. Accompanying murine and *ex vivo* studies, demonstrated that both the absence of *CFTR* gene and *P. aeruginosa* infection, had independent, adverse effects on Treg cell number and function(65).

Activation of the "classical" adaptive immune response involves antigen recognition, followed by T-cell recruitment and clonal expansion at the site of infection. Consequently, there is a time lag between the host's recognition of the presence of a pathogen and the development of an effective, adaptive immune response. In recent times, an increasing number of unconventional, "innate" T-cell populations have been described (including γ/δ, semi-invariant natural killer T [iNKT], M3 restricted T- and mucosal associated invariant T [MAIT]-cells), which are capable of mounting a more immediate response to pathogens than was previously thought possible.

γ/δ T-cells represent a minor population of circulating T-cells, which has the capacity to expand rapidly in response to bacterial infection(66). γ/δ T-cells produce IL-17 and preferentially migrate to mucosal surfaces from the circulation(67). In murine pulmonary infection models, γ/δ T-cells rapidly accumulate in the lung in response to a range bacterial pathogens, where they facilitate the influx of neutrophils and subsequent bacterial clearance(66). A single study has reported an increase in the proportion of circulating γ/δ T-cells in the blood of CF subjects, when compared to healthy subjects(68).

MAIT cells are a recently described sub-class of innate T-cells, which can be differentiated from other T-cells by the presence of an evolutionary conserved T-cell receptor (TCR) (Vα7.2-Jα33). MAIT cells recognise bacterial and fungal metabolites presented on the major histocompatibility complex (MHC) related protein-1 (MR1), however, importantly do not respond to viral stimuli(69, 70).

Innate T-cell populations provide rapid pathogen-specific responses prior to the development of classical MHC class I and II restricted T-cell responses and may also provide a sustained cytokine response in chronic infection(71, 72). However, the role of MAIT cells in CF lung disease has not been explored.

This thesis will examine the relationship between peripheral blood lymphocyte and CF phenotypes, with a focus on the MAIT cell sub-population of innate T-cells

1.3 Respiratory infection in CF

1.3.1 Epidemiology

Respiratory tract infections in people with CF begin very early life(73). Initial intermittent infections typically are caused by the common respiratory pathogens, *Staphylococcus aureus* (*S. aureus*), and non-typeable *Haemophilus influenzae* (*H. influenzae*). By adulthood the majority of sufferers have developed a chronic poly-microbial airway infection, with *P. aeruginosa* becoming the dominant pathogen in 80% of cases(1, 74). Other Gram-negative bacteria that may cause chronic infection in a minority of patients include *Burkholderia cepacia* complex (BCC), *Stenotrophomonas maltophilia* and *Acromobacter xylosoxidans*(75).

1.2.2 The airway microbiome

As bronchiectasis develops in the CF airways, a complex, poly-microbial infection develops. Traditional sputum microbiological techniques for routine CF respiratory samples use selective media to specifically culture known pathogenic bacteria, followed by determination of antibiotic susceptibility based on the minimum inhibitory concentration *in vitro*. When a single organism is responsible for pulmonary infection (e.g. community acquired pneumonia), the response to antibiotic therapy may be predicted by resulting antibiotic susceptibility profiles(76, 77). However, when treating CF pulmonary infections antibiotic susceptibility profiles do not predict clinical response(78). In part, this may be due to failure to consider the impact that non-cultured bacteria

and inter-bacterial interactions on airway inflammation and antibiotic efficacy. In an attempt to address this short-fall, culture independent (metagenomic) microbiological techniques which generate bacterial profiles based on the isolation and amplification of highly conserved bacterial-derived 16S ribosomal (r)RNA from biological samples, have been applied CF respiratory samples(79, 80). Metagenomic methods for microbial community analysis are revolutionising the understanding of a wide array of infective diseases and a number of reviews have been dedicated to this subject (including(81-83)). Application of these techniques to the study of complex infection has the potential to greatly expand our understanding of the impact of antibiotic therapy on bacterial community composition, predict responses to antibiotic combinations, and inform decisions on the optimum duration of antibiotic therapy.

To date, only a limited amount of research studies have applied metagenomic techniques to the study of CF pulmonary infection. These initial studies have demonstrated a reduction in microbial diversity with age and severity of lung disease(84, 85), and studies of lung explants also reveal significant spatial differences in community composition of airway inhabitants depending on which part of the lung is examined(86, 87).

1.2.3 *Pseudomonas aeruginosa*

P. aeruginosa is a versatile, aerobic gram-negative bacterium which is widespread in the terrestrial environment. It is an extremely robust organism and, as exemplified by its isolation from jet plane fuel and bottles of disinfectant fluid, capable of surviving in challenging and varied environmental niches(88). This adaptability is conferred by both its very large genome (~6000 genes, ~500 of which are involved in regulatory processes) and its ability to survive as either a planktonic organism, or as a member of co-dependent bacterial communities within the confines of a "biofilm"(89, 90).

The genetic plasticity and biofilm forming attributes of *P. aeruginosa* make it a highly successful pathogen in multiple disease settings in eukaryotes. In human disease, *P. aeruginosa* is a major opportunistic pathogen responsible for life threatening acute infections in burn victims and other critically ill patients, as well as causing chronic infections and acute exacerbations in patients with respiratory diseases(91-93).

In the setting of CF, establishment of a chronic pulmonary *P. aeruginosa* infection leads to decreased quality of life, and increased rate of lung function decline, pulmonary infective exacerbations, morbidity, and mortality(94).

1.2.3.1 Biofilm formation

Following initial airway infection, planktonic *P. aeruginosa* undergo rapid phenotypic and genotypic adaptation to prevent immune recognition. This is achieved by the formation of a biofilm, which offers physical protection and down-regulation of virulence factors(89, 95).

Biofilms comprise an extra-cellular matrix (ECM) of exopolysaccharides, extracellular deoxyribonucleic acid (eDNA), and proteins produced by the resident bacteria. By trapping essential nutrients and providing a physical barrier from host immune attack biofilms offer a survival advantage to embedded bacteria. In the CF lung it is proposed that *P. aeruginosa* binds abnormal mucins present in ASL to form biofilm "rafts" which float on the respiratory epithelium(95). Established biofilm infections cannot be eradicated with currently available antibiotics, or by the host's immune response(96).

Biofilm development is largely determined by its environment and available nutrients(97). *In vitro*, biofilms develop complex three-dimensional structures with phenotypically distinct sub-populations which communicate through extracellular channels(98). Iron is essential as a bacterial nutrient, but it also contributes to the structural integrity of the biofilm by crosslinking polysaccharide strands within the stalk(99).

P. aeruginosa biofilm development is dependent on cell-cell communication. Quorum sensing (QS) is a population density dependent form of communication employed by bacteria to control the synthesis of key regulatory proteins. *P. aeruginosa* employs three QS systems (Las, Rhl, and Pqs), which are integral to all activities of the bacterial community, including biofilm formation(100-103).

1.2.3.2 Shared strains

Historically, it was thought that most CF subjects become infected with a "unique" *P. aeruginosa* strain acquired from the environment, however, a number of epidemiological studies have demonstrated that groups of patients may become infected by genetically similar *P. aeruginosa*

strains, which are not found in other environmental niches(104-107). The findings of these studies raise the possibility of transmission of infection between patients. These common strains, have been described variably in the literature as “clonal”, “epidemic”, or “transmissible”, but for the remainder of the thesis will be referred to as “shared” strains(107).

In the Australian Clonal *P. aeruginosa* in CF (ACPinCF) study the relatedness of *P. aeruginosa* isolates from the sputum of nearly one thousand adults and children with CF, attending eighteen CF centres across Australia was examined(107). In this study, two thirds of subjects were infected with a *P. aeurginosa* strain seen in at least one other person, and two “major” shared strains, AUST-1 and AUST-2, were found in 22% and 18% of patients respectively(107).

Although longitudinal data assessing the clinical impact of infection with the Australian shared strains is lacking, shared strains described in other parts of the world have been associated with an increased rate of lung function decline, morbidity, mortality, and need for lung transplantation(106, 108). Similarly, limited data suggest segregating patients infected with shared *P. aeruginosa* strains may reduce cross infection(109).

Little is known about the transmissibility of shared strains, or about the phenotypic traits, which allow them to successfully infect multiple subjects. It is conceivable however, that shared strains are more adept at competing for essential nutrients within the CF lung and consequently are able thrive in this environment.

This thesis will examine the relationship between treatment strategies employed in Australian CF centres and rates of antibiotic resistant and “shared strain” *P. aeruginosa* infections

1.2.4 Current treatment strategies for pulmonary infection.

Antibiotics are used routinely to treat pulmonary exacerbations in patients with CF, yet this is based on limited evidence including only two small, randomised clinical trials(110, 111). Despite neither study providing clear evidence of improved clinical endpoints in the antibiotic treatment arms, undertaking further placebo-controlled trials would be considered unethical, given the strong anecdotal evidence for benefit.

In selecting an antibiotic regime for an individual patient important considerations include, the specific bacterial pathogens present within the airways, preferred location of treatment (hospital

versus home), route of administration, how many antibiotics to use, and which ones to prescribe (112). These decisions are often influenced by exacerbation severity, available intravenous access, previous antibiotic allergies, adherence and the support available to the individual patient if home based therapy is to be considered.

1.2.4.1 Non-*Pseudomonas* Infection.

In the absence of *P. aeruginosa* infection, the choice of antibiotics is typically based on available microbiological cultures and sensitivity patterns, and single agent treatment may be employed (Table 1.2).

1.2.4.2 *P. aeruginosa* Eradication.

The realisation that chronic *P. aeruginosa* infection results in increased lung function decline, exacerbation frequency, morbidity and mortality has led to the development of early detection programs that often involve invasive bronchoscopies and aggressive antibiotic regimes aimed at eradicating *P. aeruginosa* infection at the time of first acquisition (Table 1.3)(113-116). Implementation of these regimes within paediatric centres has proven to be highly effective in delaying the onset of chronic *P. aeruginosa* infection by several years(116), though their impact on the development of bronchiectasis is not well established(117). However, the long term impact on survival, antibiotic resistance profiles, and the potential acquisition of other opportunistic bacterial pathogens is not well understood.

Table 1-2 Oral and Intravenous Antibiotics for the Treatment of Pulmonary Exacerbations where *Pseudomonas aeruginosa* is not Isolated[#, $].

Oral Antibiotics			
Bacteria	Antibiotic	Dose	Special considerations
Methicillin sensitive *Staphylococcus aureus*	Di/Flucloxacillin	125mg BD (prophylaxis) 25mg/kg QiD (<18 years old) 1-2g QiD	Consider prophylaxis in 0-3 years old, but currently unproven clinical benefit
	Trimethorpim-sulfamethoxazole	160/800mg BD	
	Clindamycin	450mg TDS	
	Roxithromycin	150mg BD	
Stenotrophomonas maltophilia	Trimethorpim-sulfamethoxazole	160/800mg BD	Targeted treatment currently of unproven benefit
	Minocycline	200mg initial dose, 100mg BD*	
	Tigecycline	100mg initial dose, 50mg BD*	
Intravenous Antibiotics			
Methicillin Sensitive *Staphylococcus aureus*	Di/Flucloxacillin	50mg/kg (< 18years) 2-3g QID	
Methicillin Resistant *Staphylococcus aureus*	Vancomycin[$]	1g BD	
	Teicoplanin	10mg/kg BD 3 doses, then daily (< 18 years) 400mg for BD 3 doses, then daily,	
	Linezolid	10mg/kg (<12 years) 600mg daily	
Haemophilus influenzae	Cefuroxime	50mg/kg TDS (1-18years old) 1.5g TDS	
	Cefotaxime	50mg/kg TDS (1-18years old) 2g TDS	
Burkholderia cepacia complex	Ceftazadime	50mg/kg TDS (1-18years old) 2-3g TDS	A combination of at least two antibiotics from different classes should be used
	Meropenem	25-40mg/kg TDS (4-18years old) 2g TDS	
	Imipenem	22.5mg/kg TDS (<40kg) 1g TDS	
	Piperacillin-Tazobactem	90mg/kg QiD (Children) 4.5g QiD	
	Trimethorpim-sulfamethoxazole	240mg BD (6 months-6 years old) 480mg BD (6-12 years old) 960mg BD (>12years old)	
	Tobramycin[^]	10mg/kg Daily	Plasma concentration monitoring required
Stenotrophomonas maltophilia	Ticarcillin-clavulanate	80-100mg/kg QiD (<18 years) 3.1g QiD	

#: Intended as a guide only, antibiotic choice may vary dependent on local resistance profiles and antibiotic availability, $: Derived from the Report of the UK Cystic Fibrosis Trust Antibiotic Working Group, and the Australian Therapeutic Guidelines [129,130] BD: twice Daily, TDS: three times daily, QiD: four times daily kg: Kilogram, Max: Maximum dose, mg: Milligram, *: Avoid tetracyclines in patients under 12 years old, ^Therapeutic drug monitoring is required

Table 1-3 Eradications Strategies for Treatment of Specific Airway Pathogens at First Isolation[$].

Bacteria	Antibiotic	Dose	Special Instructions
Methicillin Sensitive *Staphylococcus aureus*	Flucloxacillin	100mg/kg/Day Oral	2 agents in combination for 2-4 weeks. If persistent infection consider two weeks of combination intravenous agents
	Fusidic Acid	750mg Daily Oral (>12 years old) Dosing <12 complicated see PI	
Methicillin Resistant *Staphylococcus aureus*	Topical Mupirocin	Intra-nasal	Combined use of topical Mupirocin and two other agents for 5 days
	Trimethorpim-sulfamethoxazole	160/800mg BD Oral	
	Fusidic Acid	750mg Daily Oral Dosing <12 complicated see PI	
	Rifampicin	10mg/kg/Day Oral Max 450mg <45kg Max 600mg >45kg	
Haemophilus influenzae	Amoxicillin-Clavulinic Acid	875/125mg BD Oral or 250/125mg, 2 TDS Oral Dosing <12 complicated see PI	Single agent for 2-4 weeks, repeated if still positive at end of course. Consider 2 week IV course if remains positive
	Doxycycline	200mg initial dose, 100mg BD Oral*	
	Cefaclor	500mg (>7 years), 250mg (1-7years) Oral	
Pseudomonas aeruginosa	Ciprofloxacin	15mg/kg Oral BD(under 5 years) 20mg/kg Oral BD(5-18 years) 750mg BD Oral(adult)	3weeks – 3months of ciprofloxacin combined with an inhaled antibiotic. Multiple regimens have been studied [91-94]
	Colistin	1million units BD Inhaled(under 2 years) 2million units BD Inhaled(over 2 years)	
	Tobramycin	300mg BD Inhaled	

$: Derived from the Report of the UK Cystic Fibrosis Trust Antibiotic Working Group, and the Australian Therapeutic Guidelines [129,130] BD: twice Daily, TDS: three times daily, QiD: four times daily kg: Kilogram, Max: Maximum dose, mg: Milligram, PI: Product information, IV: Intravenous, *Avoid tetracyclines in patients under 12 years old.

Table 1-4 Currently Licensed Antibiotics for the Treatment of Pseudomonas aeruginosa in Cystic Fibrosis.

Class	Drug	Route of Administration	Dose	Special Considerations
B-Lactams				
Extended Spectrum Penicillins	Ticarcillin / clavulanate[#]	Intravenous	80-100mg/kg QiD (child) 3.1g QiD (adult)	Need to be administered by slow Intravenous infusion
	Piperacillin / Tazobactem[#]	Intravenous	90mg/kg QiD (child) 4.5g QiD (adult)	
Third generation cephalosporins	Ceftazidime	Intravenous	50mg/kg TDS (Under 18 years) 3g TDS (adult)	
Carbapenems	Meropenem[#]	Intravenous	25-40mg/kg TDS (4-18 years old) 2g TDS (adult)	
	Imipenem[#]	Intravenous	22.5mg/kg Qid (under 40kg) 1g TDS (over 40kg)	Need to be administered by slow Intravenous infusion
Monobactam	Aztreonam	Intravenous	30mg/kg (under 2 years) 50mg/kg (2-12 years) 2g TDS (over 12 years)	
		Inhaled*	75mg TDS	FDA (USA) approved
Fourth generation cephalosporin	Cefepime	Intravenous	50mg/kg BD (under 40kg) 2g BD (over 40kg)	
Aminoglycosides				
	Tobramycin	Intravenous	10mg/kg Daily[$]	Plasma concentration monitoring required
	Tobramycin inhalation solution	Inhaled*	300mg BD	
Fluoroquinolones				
	Ciprofloxacin	Oral	15mg/kg (under 5 years) 20mg/kg (5-18 years) 750mg BD (adult)	Mild exacerbations and eradication regimens
Polymyxin B				
	Colistin	Intravenous	25000 units/kg TDS (under 60kg) 2million units TDS (Over 60kg)	Need to be administered by slow Intravenous infusion
		Inhaled*	1million units BD (under 2 years) 2million units BD (over 2 years)	
Other agents				
	Fosfomycin	Intravenous	100mg/kg (under 40kg) 5g TDS (over 40kg)	

mg: milligrams, kg: kilograms, QiD: four times daily, TDS: three times daily, BD: twice daily,*Have not been studied in treatment of exacerbations, # Co-administration of Probenecid may be considered to increase drug levels, $ Therapeutic drug monitoring is required

1.2.4.2 Chronic *P. aeruginosa* infection.

Once a chronic biofilm *P. aeruginosa* airway infection has become established it cannot be eradicated with currently available antibiotics and the focus of treatment moves from eradication to the treatment of exacerbations. Pulmonary exacerbations are characterised by increasing respiratory symptoms and acute deterioration of lung function. They are associated with significant morbidity and mortality and may accelerate lung function decline(118, 119), as well as being associated with diminished quality of life(120) and increased health care costs(121). In the presence of *P. aeruginosa* antibiotic therapy is usually specifically directed towards this pathogen, and modified to target co-infections if clinical response with anti- *P. aeruginosa* antibiotics is sub-optimal (Table 1.4).

Antibiotic options for treatment of *P. aeruginosa* are limited. Fluoroquinolones are the only orally administered bioavailable anti-*pseudomonal* agents. Ciprofloxacin is the most commonly prescribed quinolone, and is frequently combined with a nebulised agent in out-patient treatment regimens for mild exacerbations. Nebulisation allows delivery of high drug concentration directly to the airway whilst avoiding systemic adverse effects. Unfortunately inhaled administration of antibiotics fails to treat recalcitrant bacteria sequestered in poorly ventilated lung regions. Until recently aminoglycosides such as tobramycin and the polymyxin, Colistin were the only nebulised agents in clinical use. However, multicentre phase III randomised controlled trials (RCT's) have recently demonstrated the safety and efficacy of aerosolised Aztreonam lysine in stable patients with moderate to severe lung function impairment(122, 123), which has supported Food and Drug Administration (USA) approval for use as a maintenance treatment in CF. Despite oral and nebulised combination regimes often being used in clinical practice, there is limited evidence to support their use in the acute setting. A Cochrane Review identified three small, randomised controlled trials comparing ciprofloxacin monotherapy with combination intravenous therapy. No major differences in outcome were seen, but each of the trials were under-powered and treatment arms were not blinded (124).

Intravenous antibiotic regimes remain the cornerstone of treatment of severe exacerbations in hospitalised patients. The superiority of combination intravenous therapy for gram negative nosocomial pneumonia has been clearly demonstrated(125). However, in the setting of chronic pulmonary infection in CF, systematic reviews comparing combination with mono-therapy have failed to clearly demonstrate superiority of combination therapy(126-128). However, whilst acknowledging the current lack of evidence, treatment guidelines recommend dual anti-

pseudomomal antibiotic therapy(127, 129). No combination regimen has demonstrated clear superiority over the others, but in practice two agents from different anti-microbial classes (usually a β-lactam and aminoglycoside) are typically administered.

In selecting specific agents current practice guidelines recommend reference be made to available sensitivity profiles, and to avoid agents to which organisms are resistant(129, 130). However, there is a poor correlation between sensitivity profiles and clinical response, and an alternate approach is to select antibiotics to which the individual patient has previously responded. Anecdotally, clinicians may only refer to antibiotic sensitivity profiles when second line agents are being selected, in the situation where the patient has failed to improve with initial treatment regimen.

There are no randomised controlled trials studying the optimum duration of antibiotic therapy for pulmonary exacerbations in CF(127, 131). Theoretical concerns that a shorter duration of antibiotic therapy may reduce the time to next exacerbation, or increase the rate of lung function decline, need to be balanced against the potential adverse effects(5), and risk of promoting antibiotic resistant organisms(4) which may be associated with prolonged treatment courses.

Prospective studies into the treatment of acute exacerbations of chronic bronchitis, and microbiologically proven ventilator associated pneumonia (VAP) have demonstrated non-inferiority of shorter courses of antibiotics(132, 133). Although, of note in the case of VAP, patients infected with gram negative organisms (commonly *P. aeruginosa*) had an increased risk of microbiological relapse with shorter treatment(133).

In a small prospective cohort study of 22 CF patients undergoing hospital based treatment for pulmonary exacerbations, improvements in spirometry, and oxygenation plateaued after eight days of treatment(134). These limited prospective data are supported by a recent large retrospective study of outcomes following pulmonary exacerbations in over 1500 people, which suggested that further improvements in spirometry after eight-to-ten days of therapy were small, and that shortening antibiotic duration did not adversely affect the time to next exacerbation. Adequately powered prospective studies are required to more fully understand the optimal duration of treatment for pulmonary exacerbations in CF(135).

1.2.4.3 Effect of antibiotics on the CF airway microbiome.

Studies examining the effects of antibiotic therapy on the CF lung microbiome are limited. Tunney *et al.* have demonstrated that despite total bacterial numbers being reduced in response to antibiotics (culture-based assessment), there was relative stability in overall community composition as assessed by terminal restriction fragment length polymorphisms analysis when sputum samples were collected from subjects with CF before and after treatment of a pulmonary exacerbation(136). Daniels *et al* examined the impact of antibiotics on CF sputum microbial diversity in a group of adult CF subjects(137). Sputum samples collected following the initial 72 hours of antibiotic therapy were compared with samples collected during a period of clinical stability prior to the exacerbation and after 10-14 days of treatment. In comparison to samples collected at 72 hours, samples collected at 10-14 days demonstrated an increase in relative abundance of *Pseudomonas* species compared with non-pseudomonads, accompanied by a reduction in community diversity(137). The authors concluded that antibiotic therapy was exerting a significantly greater effect on bacterial species other than *Pseudomonas*, culminating in the dominance of *P. aeruginosa*.

This thesis will examine changes in the airway microbiome of CF patients infected with *P. aeruginosa* in response to intravenous antibiotic treatment of a pulmonary exacerbation.

1.3. Iron homeostasis in health and Cystic fibrosis

1.3.1 Systemic iron homeostasis

Iron (Fe) is an essential nutrient, critical to the oxygen carry capacity of haemoglobin and myoglobin, and as a co-factor in a multitude of metabolic pathways(138). Conversely, due to its high redox activity iron is a potential cellular toxin, capable of generating harmful reactive oxygen species (ROS) through fenton chemistry (Figure 1-1)(139). The toxic potential of iron means that tight control of iron absorption and bioavailability must be maintained to prevent harm.

Figure 1-1 Fenton Reactions.

1) $Fe^{2+} + H_2O_2 \rightarrow F^{3+} + OH^- + OH^{\cdot}$
2) $Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + OOH^{\cdot} + H^+$

With the exception of loss from denuded epithelial cells, menstrual blood, and minor incidental quantities in urine and faeces, humans do not have a mechanism of iron excretion. Consequently,

control of absorption from the gastro-intestinal tract is the sole mechanism for regulation of total body iron. Iron absorption is increased in response to iron deficiency and increased erythropoiesis, and reduced when hepatic iron stores are replete.

1.3.1.1 Hepcidin

Hepcidin, identified by three independent research groups between 1998 and 2001 has gained acceptance as the "Master Regulator" of iron homeostasis(140-142). Hepcidin is a 25 amino acid cationic peptide produced by the liver which is synthesised in response to iron loading and inflammation, and suppressed by hypoxia and anaemia(143).

Hepcidin binds to, and induces internalisation and degradation of ferroportin on the surface membrane of hepatocytes, macrophages and duodenal enterocytes. Ferroportin expression is required for export of iron from cells, consequently under conditions of iron loading, iron may be taken up by duodenal enterocytes, however, it cannot be released into the plasma, instead being retained within the cells and cleared from the body by the natural shedding of enterocytes.

Hepcidin is transcriptionally regulated, however, the mechanisms by which this is achieved are incompletely understood. Much of our knowledge of these regulatory pathways comes from the study of genetic diseases associated with iron loading and deficiency, and have been reviewed in detail elsewhere(144). In brief, hepatic cells sense increased iron concentrations through an interaction between iron-bound transferrin and the membrane bound transferrin receptors. This sensing system is dependent on HFE, which in response to high transferrin levels, relays a signal via bone morphogenic protein (BMP)-6 and its associated receptor to the SMAD pathway, which in-turn promotes transcription of hepcidin mRNA(144). Under circumstances of iron restriction or increased erythropoiesis these pathways are down-regulated and hepcidin mRNA expression decreases.

1.3.1.2 Hereditary Haemochromatosis

Hereditary haemochromatosis (HH), the most common autosomal recessive condition in Caucasian populations, impacts iron homeostasis, by inhibiting the production and release of the iron regulatory hepcidin from the liver in response to iron loading and infection(145). HH typically occurs due to mutations in the *HFE* gene and 1 in 200 Australian's carry a potentially disease causing *HFE* genotype, although phenotypic penetrance is much lower(146). Two point mutations

(C282Y and H63D) in the *HFE* gene mutations account for >90% of cases of HH and homozygotes for the C282Y mutation demonstrate the most severe phenotype, typified by increased systemic iron absorption (and decreased storage), liver cirrhosis, small joint arthropathy and diabetes mellitus(145).

This thesis we will examine whether mutations within the Haemochromatosis (*HFE*) gene serve as modifiers of CF phenotype

1.3.1.3 Iron homeostasis and infection.

Hypoferraemia is an ancient innate, anti-microbial defence mechanism, which is believed to be regulated by hepcidin. The pro-inflammatory cascade generated by infections results in the production of a host of cytokines, including IL-6. IL-6 acts on hepatocytes to stimulate hepcidin synthesis, and thereby limits circulating iron and produces a state of "functional iron deficiency". If this hypoferraemia is maintained for a prolonged period it results in decreased erythropoiesis and the development of the "anaemia of chronic disease" (ACD).

1.3.2. Clinical assessment of iron stores.

Illness may result from either insufficient (iron deficiency anaemia) or excessive (haemochromatosis) total body iron, and accurate methods of determining iron stores are important clinically. The "gold standard" test of body iron stores is quantification of stainable iron on a trephine bone marrow biopsy, however this test is invasive, painful, and impractical for population screening. Consequently, bone marrow biopsy is restricted to individual cases where accurate assessment by non-invasive means has not been possible(147). As an alternative, assays have been developed to detect the presence of proteins involved in iron homeostasis in venous blood, which may be employed as surrogate markers of iron stores.

Ferritin is the major human iron storage protein and although predominantly intracellular it may also be detected in peripheral blood. Transferrin is the major circulating, iron carrier protein in blood which may be measured, and the percentage of binding sites occupied by iron determined and expressed as transferrin saturation (TS). In health blood ferritin and TS mirror changes in whole body iron stores, being low in the iron deficient, and increasing with iron loading. However, with infection or systemic inflammation, hepcidin induced iron redistribution results in a fall in TS and

increase serum ferritin. Consequently, measuring ferritin and TS result in an over- or under-estimation of iron stores respectively in the presence of systemic inflammation(148).

Iron requiring cells express transferrin receptors (TfR) on their surface in proportion to their iron requirements. A proportion of these TfR are non-selectively cleaved from the surface of the cells and are detectable in blood as soluble transferrin receptors (STfR). The STfR concentration is proportional to the cellular "need" for iron and increases in response to iron deficiency. STfR concentration is unaffected by the acute phase response and has advocated for assessment of iron status in the presence of systemic inflammation(149). In the setting of chronic disease, differentiation between the anaemia of chronic disease and iron deficiency anaemia is challenging, and the two conditions may co-exist. Working with the premise that ferritin is the best indicator of whole body iron stores, and the STfR most reliably reflects functional iron requirements, the STfR/Log_{10}ferritin ratio (STfR ratio) has been proposed and validated as a mechanism of detecting the presence of iron deficiency in situation where the ACD may be present(150-152).

1.3.3 Lung iron homeostasis

The combination of high oxygen concentration and appreciable amounts of catalytically active iron in atmospheric particulate matter provides the lung with unique challenges with regards to iron homeostasis(153). Airway cells must rapidly sequester and detoxify iron to prevent both damage from the generation of free radicals, as well withhold this key nutrient from invading pathogens.

The lung employs similar iron homeostatic mechanism as the gastro-intestinal tract to detoxify the airways, although their regulatory mechanisms appear different(154). The iron binding protein lactoferrin is a member of the anti-microbial *defensins* produced by neutrophils and submucosal serous cells of the lungs. Lactoferrin is released into ASL in response to inflammation and iron loading, where it chelates unbound iron, and modulates the innate immune response by suppressing the generation of reactive oxygen species and binding microbial derived pro-inflammatory cytokines(155, 156). Iron laden lactoferrin binds to the lactoferrin receptor, which is in-turn internalised and delivers complexed iron to ferritin(157). Ferritin is present in high concentrations in airway epithelial cells, and may be exported to the airway surface liquid or plasma to prevent excessive accumulations within cells(154). Ferroportin and transferrin also contribute towards iron export from pulmonary epithelial cells(158).

AEC's are also capable of divalent metal transporter (DMT)-1 receptor mediated uptake of unbound iron and increase DMT-1 expression in response to iron loading through increased transcription of an iron responsive element (IRE) negative isoform of the protein(159). DMT can only bind ferrous iron, consequently iron is first reduced by duodenal cytochrome-b (DYCTB) or O_2^- (produced by anion exchange proteins, which facilitate electron-neutral exchange of Chloride and bicarbonate anions at the apical cell membrane)(160, 161).

Finally AM's contribute to iron detoxification in the lung through natural resistance-associated macrophage protein (NRAMP)-1 (analogous to DMT-1) mediated phagocytosis of iron containing particulate matter(162).

1.3.3.1 Iron Homeostasis in pulmonary disease.

There is an abundance of associational data to suggest that ineffective iron handling within the lung is associated with predisposition to a number of acute and chronic pulmonary diseases(157).

Acute respiratory distress syndrome (ARDS) is typified by an intense parenchymal inflammatory reaction within the lungs in response to a pulmonary, or extra-pulmonary insult, and results in severe hypoxaemia. Increased serum ferritin and BALF iron content have each been associated with the risk of development, and severity of ARDS(163, 164). In addition polymorphisms in iron handling genes have been linked with an increased risk of developing ARDs in response to an appropriate insult(165).

The risk of pulmonary *Mycobacterium tuberculosis* (MTB) has similarly linked with the increased dietary iron intake in endemic regions(166). In these studies, it has been proposed that iron overload within AM's impairs mycobacterial killing. However, the co-existence of Human immunodeficiency virus infection (HIV) in many of the subjects included in these studies makes the results difficult to interpret.

Defective iron handling has also been implicated in the pathology of toxin induced fibrotic lung diseases, pulmonary alveolar proteinosis, and lung transplant rejection(157).

1.3.4 Iron Homeostasis in CF

1.3.4.1 Systemic iron stores in CF

Anaemia occurs in up to 30% of subjects with CF, with true iron deficiency and the ACD being reported as common causes(167). Chronic inflammation, pulmonary haemorrhage and malabsorption occur commonly in CF, and through their impact on hepcidin release, can each effect iron homeostasis(168).

Iron deficiency rates in excess of 60% have been reported in adult subjects with CF, however, as discussed previously accurate assessment in the setting of systemic inflammation is difficult, and rates of decreased total body iron stores are likely significantly less than this(169, 170). It is possible iron indices which are unaffected by inflammation may be of greater value in determining "true" iron deficiency(170). However, a recent study has suggested STfR is insensitive for the detection of iron deficiency in children with CF(171).

Accurate assessment of iron stores is important for guiding the need for iron replacement therapy. In CF this is of particular relevance, as theoretical concerns exist that injudicious administration of iron supplements may override innate iron withholding mechanism and promote infection and inflammation(167, 172).

In support of the hypothesis that iron handling may be important in CF, several authors have suggested mutations in the *HFE* gene may modulate CF phenotype(173-175). In two studies, performed in children with CF, a trend towards an increased risk of bowel obstruction was reported in subjects who carried a C282Y *HFE* mutation(173, 175). Furthermore, a single centre study, of a heterogeneous group of adults and children demonstrated lower absolute lung function values and increased rate of forced vital capacity (FVC) decline, as well as an increased risk of gastrointestinal complications and diabetes in subjects who carry a *HFE* gene mutation(174).

1.3.4.2 Pulmonary iron homeostasis in CF

Respiratory secretions and sputum from subjects with CF contain micromolar concentrations of iron, making this micronutrient more readily available to inhaled pathogens (airway iron indices from existing studies are presented in Table 1.5)(176-178). In this setting, iron content correlates

with the concentration of the iron regulatory cytokines Interleukin (IL)-1β and tumour necrosis factor alpha (TNF-α), which in turn stimulate ferritin and transferrin production(177). Furthermore, iron concentrations positively correlate with *P. aeruginosa* colony counts in the sputum of patients with CF confirming its importance *in vivo*(179).

Table 1-5 Studies reporting iron concentration in respiratory secretions from patients with Cystic fibrosis.

Author	Year	Population	Controls	Substrate	Assay	Iron concentration Controls	Iron concentration CF
Gifford AH *et al* [160]	2011	Adults	None	Expectorated sputum	Inductively coupled plasma mass spectrometry	Not available	Stable 1.11 mg/ml (0.09-4.01)* Exacerbation 2.22 mg/ml (0.77-7.04)*
Reid DW *et al* [172]	2007	Adult and Paediatric	Healthy	Expectorated sputum	Colorimetric	0 µmol/Litre (0-15.8)*	*P. aeruginosa* infection 34 µmol/Litre (2.4-78)* No *P. aeruginosa* infection 18 µmol/Litre (8-118)*
Reid DW *et al* [170]	2004	Adult	Healthy	Expectorated sputum	Colorimetric	0 µmol/Litre (0-13.2)*	Stable 33.3 µmol/Litre (0-111.2)* Exacerbation 44.4 µmol/Litre (17.0-128.7)*
Stites S *et al* [169]	1999	Adult	Healthy non-smokers	BAL	Colorimetric	0 µg/dl#	42 µg/dl (11.6)#
Stites S *et al* [171]	1998	Adult	Non-smokers recent URTI	Expectorated sputum	Coulometry	0 ng/mg#	242 ng/mg (47)#

CF: Cystic fibrosis, URTI: Upper respiratory tract infection, BAL: Bronchoalveolar lavage, µmol: Micromoles, µg: Micrograms, dl: decilitre, ng: nanograms, mg milligrams, * Median and range, # Mean and standard deviation.

In vitro studies have demonstrated DF508del CFTR mutant human AEC's contain increased intra-cellular iron and "leak" excessive iron to their apical surface, which in turn promotes the growth of *P. aeruginosa* biofilms on the apical cell surface(180). Furthermore, patients frequently have frank blood in their sputum and sub-clinical bleeding into the airway is probably common(181).

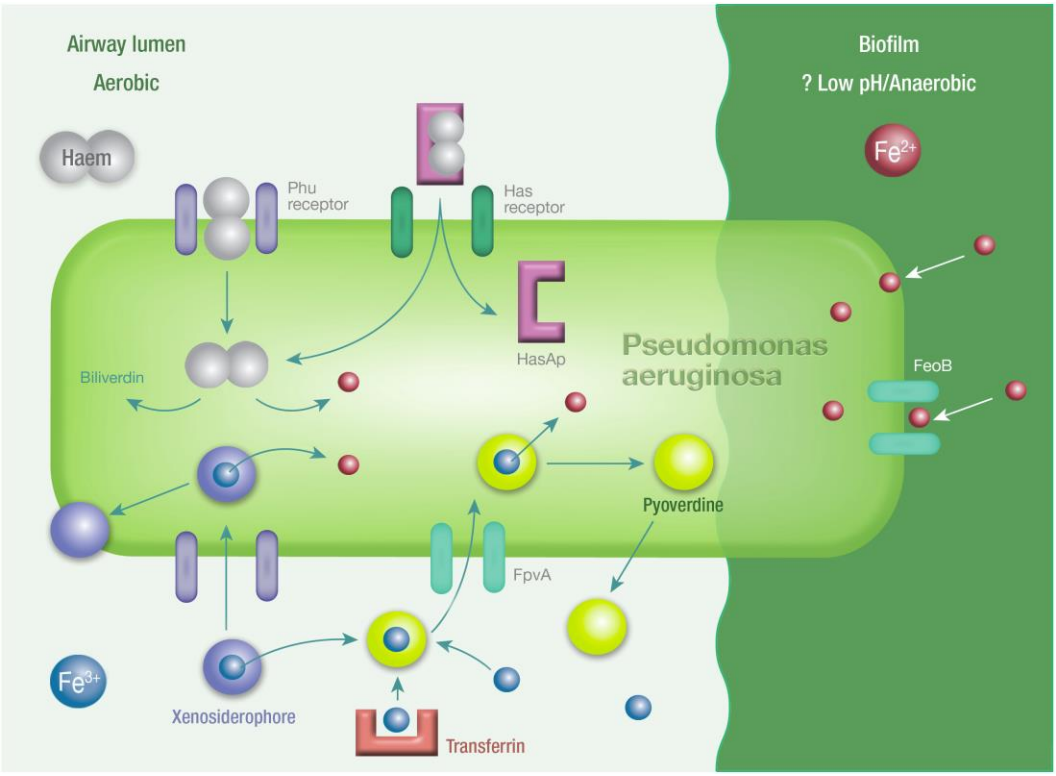
The combination of the *in vitro* and *in vivo* work support a hypothesis that iron handling in the CF lung is intrinsically defective and results in increased availability of bioactive iron in the lung, which may potentially "feed" chronic infection and promote oxidative damage of host tissues.

1.3.5 Iron homeostasis in *P. aeruginosa*.

P. aeruginosa is intrinsically reliant on iron for survival and 6% of its transcribed genes are iron-responsive(182). The concentration of bioavailable iron is a powerful regulator of *P. aeruginosa* behaviour, influencing inter-cellular communication and biofilm formation(182).

P. aeruginosa may take up iron from either haem or non-haem iron sources (Figure 1.2). Two haem uptake systems have been described in *P. aeruginosa* (Phu and Has)(183). The Phu system relies on direct binding of haem or haem-containing proteins to a membrane bound receptor, whereas the Has system secretes a haem binding protein (HasAp) which is reabsorbed through the Has Receptor (HasR) when bound to haem(184, 185). The *P. aeruginosa* genome contains a third haem receptor encoding gene (*hxuC*), however, its functional regulation has yet to be characterised(186).

Figure 1-2 *Pseudomonas aeruginosa* iron acquisition pathways.



Free or transferrin bound ferric (III) iron (blue spheres) binds to either *P. aeruginosa* specific (e.g. pyoverdine [green spheres]) or xeno-siderophores (purple spheres) and is taken up through siderophore specific channels. In anoxic lung regions, it is postulated that limited amounts of Ferrous (II) iron (red spheres) is present that can be taken up directly by binding to FeoB surface receptors. Finally direct uptake of haem (double grey spheres) may occur directly through Phu receptors, or by first being bound to HasAP before uptake via and Has receptors.

Haem is an uncommon iron source in the natural environment and *P. aeruginosa* must also be capable of scavenging non-haem iron, which in aerobic environments is present in the insoluble ferric (Fe^{3+}) form. *P. aeruginosa* produces high affinity ferric-iron chelating siderophores(187). Siderophores are secreted by *P. aeruginosa* into the local environment to chelate free iron and "strip" iron from host iron binding proteins.

Two distinct siderophores have been characterised in *P. aeruginosa*; pyoverdine and pyochelin. Over fifty distinct pyoverdine sub-types have been described and they are responsible for the distinctive yellow-green fluorescence of certain Pseudomonads(188).

Pyoverdines are the primary siderophore produced by *P. aeruginosa*, with one of three distinct sub-classes being produced by individual strains(189).

Pyochelin is considered a secondary siderophore in *P. aeruginosa* having a much lower iron binding affinity than pyoverdine(186, 187). Pyochelin appears to have less influence on the biofilm forming capacity of *P. aeruginosa* than pyoverdine, and its importance for iron acquisition during clinical airway infections is unclear(187, 190). In addition to acquiring iron using autologous siderophores, *P. aeruginosa* has a high capacity to take up iron-laden siderophores produced by other bacteria and fungi(186).

P. aeruginosa is naturally an aerobic bacterium, but it is highly capable of adaptating to survive in the low oxygen environment encountered within plugged CF airways. Within these regions of low oxygen tension and low pH there is potential for the redox status of Fe to change to the more "soluble" ferrous (Fe^{2+}) form. Ferrous iron may be acquired by *P. aeruginosa* by passive diffusion or uptake through the FeoB receptor, although the role of these mechanisms in the clinical setting is unclear(191).

P. aeruginosa iron acquisition systems are tight controlled by the ferric uptake regulator (Fur). Fur acts both directly, and indirectly through extra-cytoplasmic sigma factors (including PvdS) to limit iron absorption(192). Under iron-replete conditions, Fur binds ferrous iron and attaches to a consensus sequence (Fur-Box) in the promoter region of genes instrumental in iron acquisition thus suppressing their transcription(193). In the presence of iron, Fur inhibits "iron conservation" strategies by suppressing the production of two small RNA's (PrrF1 and PrrF2)(194). In the absence of iron, these sRNA's are synthesised and facilitate inhibition of genes which encode "non-essential" iron-containing proteins thereby maintaining the cytoplasmic iron pool for essential use(195). In low iron environments siderophore synthesis increases and non-essential iron consuming processes are down-regulated. Several excellent comprehensive reviews of the iron acquisition systems employed by *P. aeruginosa* have recently been published(186, 187, 190, 192, 195), but the above overview highlights the central role of iron in *P. aeruginosa* biofilm development.

This thesis will examine the relationship between the concentration of iron and other bio-active metals in the airway secretions of patients with CF and the severity of airway inflammation and lung disease

1.3.6 Targeting *P. aeruginosa* iron acquisition as novel therapeutic strategy

The critical role of iron in *P. aeruginosa* survival and biofilm formation may represent a potential "Achilles Heel" in the defensive armamentarium of this fastidious pathogen. Thus considerable research endeavours on a variety of fronts are being undertaken to develop novel therapeutic strategies based on disruption of bacterial iron homeostasis.

Proposed therapeutic strategies include: i) delivery of toxic amounts of iron to biofilm dwelling bacteria, ii) blocking iron dependent metabolic pathways with iron or siderophore mimetics, iii) use of high affinity iron chelators to "starve" bacteria of iron and iv) development of vaccine which target highly conserved iron uptake receptors(196). Research in each of these areas is currently at the stage of *in vitro* or early pre-clinical study stage. Iron targeted therapies are discussed in detail in the published review manuscript, which is included as part of this thesis submission (Appendix 1 - Targeting iron uptake to control *Pseudomonas aeruginosa* infections in cystic fibrosis).

1.4 Thesis Aims and Objectives

1.4.1 Background and Scope.

Despite the high initial success of eradication regimens in delaying acquisition, the majority of CF subjects will eventually develop a chronic *P. aeruginosa* infection(116, 197-199). Establishment of *P. aeruginosa* infection is a sentinel event in the life of patients with CF and is associated with increased rates of pulmonary exacerbations, accelerated lung function decline, reduced quality of life and poorer survival(200, 201). The importance of *P. aeruginosa* is recognised in consensus statements for the treatment of pulmonary infection in CF(94, 127, 202). Furthermore, the emergence of shared (and likely transmissible) *P. aeruginosa* strains in many CF clinics, is likely add further complexity to the treatment and cohort segregation in the future(107).

The expansion of health information systems has allowed data to be collected and collated on large populations of patients and CF Data Registries are now available in many parts of the world(1, 3, 203, 204). Utilisation of data collected through registries has enabled between-centre comparisons to identify variations in clinical outcomes between CF centres and has led to the establishment of quality improvement programs, including benchmarking of outcomes with "high-performing" centres(203-205). Studies based on large patient registries and databases have suggested more aggressive use of antibiotics to treat clinical deterioration results in better clinical outcomes for children and adults with CF(203, 206-208). A recent analysis of the CF Foundation Benchmarking Project, identified characteristics of CF centres (paediatric and adult) achieving outstanding clinical outcomes which included: a) strong leadership and a well-functioning care team working systematically to provide consistent care; b) high expectations for outcomes by the healthcare team and families; c) early and aggressive management of clinical deterioration and d) engaged and empowered patients/families who were well informed on CF management and its rationale(203). The high performing teams identified in this project had a low threshold for aggressive treatment of clinical decline, which resulted in high levels of use of antibiotics for pulmonary symptoms.

However, to date, few studies have used national datasets to assess the variability and impact of antibiotic treatment strategies for *P. aeruginosa* in CF patients. The generation of antibiotic resistance in *P. aeruginosa* by aggressive antibiotic treatments runs the risk of adverse clinical outcomes later in the natural history of CF lung disease and paradoxically may contribute to persistence. Consequently, the first **aim** of this thesis was to utilise data from the ACPinCF study(107) and the Australian CF Data Registry (ACFDR) to examine differences in managing *P. aeruginosa* infection and determine the relationship between treatment intensity and antimicrobial resistance of *P. aeruginosa* in the Australian CF population.

Established *P. aeruginosa* pulmonary infection cannot be cleared by currently available antibiotics and new therapies are required to tackle chronic infections. The development of new therapeutic strategies is reliant on a good understanding of the environmental, host and bacterial factors that may contribute towards the persistence of infection.

Traditional sputum microbiological techniques are capable of culturing only a limited number of the bacteria present in established bronchiectasis, and antibiotic susceptibility profiles generated by conventional single agent antibiotic testing, do not correlate with response to treatment(209, 210). In response, techniques to test the sensitivity of *P. aeruginosa* to combinations of antibiotics have been developed(78). However, again, this approach has had limited success, with similar clinical and bacteriological responses observed in patients receiving treatment based on single agent sensitivity testing results, compared to multiple combination bactericidal testing, in a randomised controlled trial(78, 211).

The discrepancy between the results of *in vitro* susceptibility testing results and clinical response are probably attributable to factors not accounted for in currently used susceptibility testing strategies including: i) environmental factors present in the CF lung not replicated *in vitro* (e.g. pH, oxygen and nutrient availability), ii) mode of growth (e.g. planktonic *versus* biofilm) and iii) bacterial synergy in a polymicrobial infection.

As pulmonary disease progresses, plugging of the distal airways by dehydrated sputum, creates micro-aerobic or frankly anaerobic pockets, with low pH and altered nutrient availability(212, 213). The highly abnormal environment within these regions drives phenotypic adaptation of bacteria. Furthermore, non-bacterial factors within this milieu may directly contribute towards local tissue destruction by generating reactive oxygen species and proteolytic enzymes(214). Bio-active trace metals (biometals) are essential co-factors in a wide range of human and bacterial enzyme systems, however, strict regulation of their bioavailability is essential to prevent toxicity(215). A limited number of previous studies in patients with CF, non-CF bronchiectasis and chronic bronchitis have demonstrated increased iron, zinc and copper concentrations in airway secretions, and have postulated that these metals may influence disease severity(179, 216). *In vitro* studies demonstrate that iron strongly influences the ability of *P. aeruginosa* to form biofilms and manipulation of iron availability has been proposed as a novel therapeutic strategy for the treatment of chronic *P. aeruginosa* infection(196, 217). An observational study by Gray and colleagues, demonstrated increased concentrations of zinc and copper in the CF lung(216), and in separate *in vitro* studies these metals have been shown to induce *P. aeruginosa* resistance to carbapenem antibiotics(218). The origin of airway metal ions has not been determined, with potential sources including vascular leak, channelopathies and release from necrotic airway cells, or the bacteria themselves.

Emerging, culture independent microbiological (metagenomic) techniques, aim to more completely characterise the microbiome of poly-microbial infections. These techniques are based on the amplification of highly conserved, bacterial-derived 16S rRNA from biological samples(209). Generated sequences reads are then compared to established bacterial DNA databases to allow bacterial identification(219). It is anticipated that the application of metagenomics to the study of the lung microbiome will provide novel information on how the microbiome responds to currently applied antibiotic regimens, and inform the development of more successful treatments in the future.

The intrinsic susceptibility of the CF lung to infection, supports supposition that immune responses in CF are defective(25). At the level of the airway lumen, the cellular immune response is dominated by activated neutrophils. However, in contrast, airway epithelial biopsies demonstrate a profound T-cell infiltrate, supporting an important role for adaptive immune responses in the orchestration of a sustained inflammatory response(47). To date, studies of immune responses in CF have tended to concentrate on innate immune and neutrophil responses, and there has been less consideration of adaptive immune responses.

Research into adaptive immune responses in CF have largely focused on the classic dichotomy of T-helper (Th)-1 and Th-2 responses(49). These early studies suggested a skew towards a Th-2 response in most CF subjects with *P. aeruginosa* infection, which resulted in increased pulmonary inflammation and disease progression(49-51). Subsequently, the identification of high levels of IL-17 in BALF, combined with the presence of large numbers of IL-17 positive T-cells in airways biopsy from people with CF, resulted in postulation that CF lung disease may be Th-17 mediated(55-58). Innate T-cells, are capable of providing an immediate response to infection and a number of lineages can also produce large quantities of IL-17(67, 71, 220). However, to date the role of innate T-cells in defending the CF lung from established infection have not been considered.

Based upon the above observations, the next broad **aim** of this thesis was to determine bacterial and host factors that may influence pulmonary infection and response to antimicrobial therapy, with a specific focus on innate T-cell responses, iron and other biometal content of airways secretions, and how the whole lung microbiome responds to currently administered intravenous antibiotics.

The pulmonary phenotype is variable between individuals with similar CF genotypes. In part, this variability may reflect different environmental exposures and quality of clinical care provided, however, the influence of non-*CFTR* gene modifiers has also been postulated(18, 25). Genes that encode immune mediators, anti-oxidants, ion transporters and pancreatic β-cell function have each been implicated in modifying CF phenotype(18, 20, 22-24).

Recognising the potential for iron to generate ROS in the airway and "feed" bacterial infection(139, 179), led to the hypothesis in this thesis that mutations in genes involved in iron homeostasis may influence CF phenotype(175).

Mutations in the *HFE* gene increase the risk of excessive systemic iron absorption and result in abnormal iron handling at the cellular level(146, 221). Furthermore, earlier studies have suggested *HFE* mutations may result in a more severe CF phenotype, however, these studies have been either underpowered, or have been performed in heterogeneous groups of patients(173-175).

Consequently the final broad **aim** of this thesis was to examine how iron handling and *HFE* genotype may modify the CF phenotype.

1.4.2 Broad Research Aims

The Research within this thesis was based on the overarching aims to:

i) Determine the relationship between treatment practices in Australian CF centres and rates of *P. aeruginosa* antibiotic resistance.

ii) Determine host and bacteria related factors which may influence pulmonary infection and response to anti-microbial therapy.

iii) Determine the relationship between systemic and pulmonary iron homeostasis and CF lung disease.

1.4.3 Specific Research Aims

The specific aims of the research within this thesis were to:

i) Investigate the relationship between treatment intensity and the antimicrobial resistance of *P. aeruginosa* in the Australian CF population.

ii) Determine the relationship between shared *P. aeruginosa* strains and antimicrobial resistance and treatment intensity in the Australian CF population.

iii) Explore the effects of intravenous antibiotics, administered to treat an infective pulmonary exacerbation, on the CF lung microbiome.

iv) Determine whether the lymphocyte sub-sets are different in subjects with CF compared to healthy controls.

v) Determine the association between lymphocyte sub-sets and disease phenotype in subjects with CF.

vi) Explore the bio-active metal composition of airway secretions in health and CF airways disease.

vii) Examine the association between airway bio-active metals, airway inflammation and outcomes in CF.

viii) Explore whether mutations in the *HFE* gene have a modifying effect on CF phenotype

Chapter 2. Trends in P. aeruginosa antibiotic resistance in Australian CF centres.

2.1 Abstract

Introduction: In cystic fibrosis (CF), chronic *Pseudomonas aeruginosa* infection is associated with increased morbidity, antibiotic treatments and mortality. By linking Australian CF registry data with a national microbiological dataset we examined the association between treatment intensity and *P. aeruginosa* antibiotic resistance.

Methods: Sputa were collected from paediatric and adult CF patients from 18 Australian CF centres. *P. aeruginosa* antibiotic susceptibilities determined by local laboratories were correlated with clinical characteristics, treatment intensity and infection with strains commonly shared amongst Australian CF patients. Between-centre differences in treatment and antibiotic resistance were compared.

Results: Large variations in antibiotic usage, maintenance treatment practices and multi-antibiotic resistant *P. aeruginosa* (MARPA) prevalence exist between Australian CF centres, although overall proportions of MARPA isolates were similar in paediatric and adult centres (31% versus 35%, p=0.29). Among large paediatric centres MARPA correlated with intravenous antibiotic usage and centre, while inhaled antibiotics, reduced lung function and centre predicted intravenous antibiotic usage. In large adult centres, body-mass index and centre correlated with MARPA, while intravenous antibiotic use was predicted by gender, centre, inhaled antibiotics and lung function. *P. aerug*inosa strains AUST-01 and AUST-02 independently predicted intravenous antibiotic usage and MARPA.

Conclusion: Increased treatment intensity in paediatric centres is associated with greater risk of MARPA, but not worse clinical outcomes in adults or children. Innovative approaches to chronic *P. aeruginosa* infection are needed to optimise the benefits of early and aggressive therapy, while minimising treatment-related morbidity.

2.2 Introduction

In cystic fibrosis (CF) chronic *Pseudomonas aeruginosa* infection is associated with accelerated pulmonary decline, reduced quality of life, increased treatment burden and poorer survival.[200, 222] CF consensus statements therefore emphasise the importance of managing *P. aeruginosa* pulmonary infections.(127, 202) However, commonly shared and multi-antibiotic resistant *P. aeruginosa* (MARPA) strains emerging in CF centres worldwide add further complexity to patient management and infection control policies.[223]

Utilising data collected by national CF data registries allows between-centre comparisons and quality improvement programs to benchmark against "high-performing" centres.(203-205) However, few studies have assessed *P. aeruginosa* treatment strategies using national datasets. Here, data from the Australian Clonal *Pseudomonas aeruginosa* in CF (ACPinCF) study[107] and the Australian CF Data Registry (ACFDR) were linked to examine differences in managing *P. aeruginosa* infection. The aim was to explore in the Australian CF population the association between treatment intensity and *P. aeruginosa* antibiotic resistance.

2.3 Methods

2.3.1 Patients, data and sample collection

The ACPinCF study involved 18 Australian CF centres and is described in detail elsewhere.[107] Briefly, it examined the point prevalence, diversity and clinical impact of *P. aeruginosa* strains in Australian CF clinics, and included 2,677 patients (1,300 aged ≥18-years; centre size: 25-294), representing 91%, 89% and 90% of the paediatric, adult and total Australian CF population respectively.(224)

Human Ethics Committees of all participating institutions approved the study. Participants provided written, informed consent, which for children included parents or legal guardians.

Each patient provided a single sputum specimen either during a routine clinic visit or during hospitalisation between September 2007 and June 2010. Age, gender, inhaled maintenance therapies (tobramycin, colistin, dornase-alpha and hypertonic saline) and oral azithromycin in the previous 30-days; and number of courses and total days of intravenous antibiotics, and

out-patient clinic visits during the previous 12-months were recorded. The best forced expiratory volume in one-second (FEV_1) in the calendar year of sample collection (and forced vital capacity [FVC] and body-mass index [BMI] from the same day) reported to the ACFDR were also recorded. In children, age-adjusted pulmonary function prediction equations[225, 226] and standard deviation (SD) z-scores for BMI were utilised.[227]

2.3.2 Laboratory testing

Sputum culture and antibiotic susceptibility testing were performed routinely by hospital laboratories. Six centres performed sensitivity testing on a "mixed" colony sweep of culture plates, while in the remaining 12 individual "pure" colonies were selected for testing.[228] Disk diffusion sensitivity testing was applied by all centres, 12 used Clinical and Laboratory Standards Institute (CLSI) guidelines, five the Continuous Dichotomous Susceptibility (CDS) test, and one applied European Committee on Antimicrobial Sensitivity Testing (EUCAST) methods and breakpoints(229-231) (Table 2.1). Three *P. aeruginosa* colonies representing different morphotypes from each specimen were selected locally and transported to the research laboratory to confirm their identity[232] and for enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) genotype testing.[107]

MARPA isolates were defined as being resistant to all antibiotics tested in two or more classes of anti-pseudomonal agents (β-lactam, fluoroquinolone and aminoglycoside antibiotics).(233) Where antibiograms for multiple isolates from the same sample existed (398/934 samples), the most resistant isolate was used. If an isolate's susceptibility to an antibiotic class was not tested, it was excluded from analysis of resistance to that antibiotic class, and if not already identified as a MARPA, from this category too. Thus, 19 isolates were excluded from the MARPA analysis because of lacking data for aminoglycoside (n=1), β-lactam (n=2) and fluoroquinolone (n=21) susceptibilities.

2.3.3 Shared *P. aeruginosa* strains

Shared *P. aeruginosa* strains had indistinguishable ERIC-PCR gel patterns from sputum isolates cultured from at least two patients as described previously.[107] To explore their influence on antibiotic resistance, differences between patients infected with the two most prevalent Australian shared strains (AUST-01 and AUST-02) and those with unique strains

were examined. To increase the likelihood that antibiotic susceptibility profiles from local laboratories reflected strains genotyped by the reference laboratory, this analysis was limited to patients with the same ERIC-PCR genotype for each of the three isolates typed.

2.3.4 Between-centre comparisons

Between-centre differences in treatment and outcome were conducted by selecting the largest paediatric and adult centre in each state. This helped minimise data skewing from small centre effects. Each of the analysed States is identified by the letter, A-to-F, with the suffix (p) or (a) denoting paediatric and adult centres respectively.

Table 2-1 Site specific antibiotic susceptibility testing methods and % of samples tested against individual agents.

				Percentage of Isolates tested against each antibiotic												
Site	Technique	Strain Selection	Reference Range	Gentamicin	Tobramycin	Amikacin	Ceftazadime	Cefepime	Ticarcillin/ Clavulanate	Piperacillin	Piperacillim/ Tazobactam	Aztreonam	Meropenem	Imipenem	Ciprofloxacin	Colisitin
A(p1)	Disk	Mixed	CLSI	100	91	90	93	0	100	9	0	23	77	0	79	28
A(a2)	Disk	Pure	CLSI	100	97	99	99	3.5	100	96	1	99	97	0	97	99
A(p3)	Disk	Mixed	CLSI	100	100	100	100	0	100	0	0	0	100	0	100	0
A(a4)	Disk	Mixed	CLSI	100	100	100	100	0	94	0	0	0	97	0	100	0
A(a5)	Disk	Pure	CLSI	100	100	100	100	100	100	87	40	100	100	0	100	93
A(p6)	Disk	Mixed	CLSI	100	100	100	100	100	100	75	50	100	100	0	100	100
B(p1)	Disk	Pure	CDS	100	100	94	94	83	100	100	6	0	83	83	89	0
B(a2)	Disk	Pure	CLSI	100	0	100	0	100	100	0	0	0	100	0	100	0
B(p3)	Disk	Mixed	CDS	100	100	100	100	100	100	0	3	100	100	100	100	0
B(a4)	Disk	Pure	CDS	100	90	90	85	80	100	85	0	45	85	5	100	5
B(p5)	Disk	Pure	CDS	100	39	39	23	39	100	31	0	8	23	0	100	0
C(p1)	Disk	Mixed	CLSI	100	100	100	100	100	100	100	100	97	100	0	100	0
C(a2)	Disk	Pure	EUCAST	1	99	1	98	0	98	0	1	1	98	0	98	1
C(a3)	Disk	Pure	CLSI	100	100	100	100	100	100	100	100	0	100	0	100	96
D(a1)	Disk	Pure	CLSI	100	100	100	100	0	0	100	0	0	100	0	100	0
D(p2)	Disk	Pure	CLSI	100	100	0	100	0	0	100	0	100	100	0	100	100
E(a1)	Disk	Pure	CLSI	100	99	98	0	99	100	0	99	99	99	0	100	98
F(a1)	Disk	Pure	CDS	100	58	58	100	0	100	0	0	84	0	58	100	0

Disk: Disk diffusion sensitivity testing, Pure: Isolate selected from the plate for sensitivity testing, Mixed: Sweep taken from the plate for sensitivity testing, CDS: Continuous Dichotomous Susceptibility. CLSI: Clinical and Laboratory Standards Institute; EUCAST: European Committee on Antimicrobial Sensitivity Testing; Site A(a/pX): Denotes a treatment centre in State A-F (adult/paediatric)

2.3.5 Statistical Analysis

Data analysis was performed using PASW, Version 18.0 (SPSS Inc. Chicago, IL, USA). Between-group differences of continuous variables were analysed by t-tests, analysis of covariance, Mann-Whitney or Kruskal-Wallis tests according to the normality of the data distribution. Correlations were examined by Spearman's Rho and categorical variables by Chi-squared tests. Multi-logistic and linear-regression analyses were performed to identify independent predictors of MARPA and intravenous antibiotic usage in the previous 12-months respectively. To determine the impact of shared strains, regression models were re-run on a sub-group of 574 subjects harbouring either AUST-01, AUST-02 or a unique *P. aeruginosa* strain, with the variable *P. aeruginosa* strain type incorporated. State A (coordinating State for ACPinCF) acted as the reference state in all models. A P-value <0.05 was considered statistically significant.

2.4 Results

Antibiotic resistance data were available for at least one isolate from 934/983 (95%) patients (228 children) culturing *P. aeruginosa* from their sputum. Forty-eight percent of patients were F508del homozygotes, 32% F508del heterozygotes, 3% had other mutations and 17% had unknown CF genotypes. The clinical characteristics and isolate antibiotic resistance profiles of subjects treated in the major paediatric and adult centre in each state are summarised in Tables 2.2A and 2.3A and for all treatment centres included in the study, in Tables 2.2B and 2.3B.

Table 2-2 Comparison of clinical characteristics, treatment burden and *Pseudomonas aeruginosa* resistance profiles of patients attending each Australian paediatric centres included in the study.

Part A. The major paediatric treatment centre in each state.

	Whole Cohort (n=228)	State-A(p1) (n=57)	State-B(p3) (n=31)	State-C(p1) (n= 62)	State-D(p2) (n=23)	P-value[‡]
Age	14 (11-17)	14 (12-16)	13 (12-15)	14 (9-17)	17 (15-19)	**0.001**
Males, number (%)	114 (50)	25 (44)	15 (48)	34 (55)	16 (70)	0.19
FEV_1 % predicted*	74 (55-88)	79 (55-90)	70 (60-81)	68 (53-87)	73 (46-87)	0.75
FVC % predicted*	88 (70-98)	89 (68-100)	87 (77-98)	87 (68-97)	88 (71-96)	0.93
BMI z-score*	-0.35 (-0.96 – 0.23)	-0.16 (-0.75-0.35)	-0.72 (-1.1- -0.16)	-0.32 (-1.00-0.36)	-0.02 (-1.32-0.28)	0.06
No. of outpatient visits*	6 (4-8)	7 (4-9)	5 (2-7)	6 (4-7)	7 (5-7)	0.13
No. of IV antibiotic courses*	1 (0-3)	1 (0-3)	1 (0-2)	1 (0-2)	3 (1-4)	**0.001**
No of IV antibiotic days*	14 (0-35)	15 (0-37)	14 (0-23)	10 (0-23)	55 (19-81)	**<0.001**
% Inhaled antibiotic[†]	46	35	65	54	67	**<0.05**
% Dornase alpha[†]	56	63	65	46	81	**<0.05**
% HTS[†]	29	39	13	9	77	**<0.001**
%Azithromycin[†]	54	67	36	37	73	**<0.01**
Antibiotic susceptibility testing methodology	-	CLSI	CDS	CLSI	CLSI	
P. aeruginosa antibiotic resistance, %						
- Aminoglycosides	43	56	32	24	74	**<0.001**
- β-lactam agents	21	30	7	5	61	**<0.001**
- Fluoroquinolones[§]	30	49	26	16	39	**0.002**
- MARPA[¶]	31	55	16	10	65	**<0.001**

Part B. Other paediatric treatment centres included in the study

	State A(p3)	State A(p6)	State B(p1)	State B(p5)
Participants, n (% of total clinic population)	20 (20)	4 (16)	18 (12)	13 (20)
Age	14 (11-16)	12 (6-17)	15 (11-16)	14 (11-17)
Males, number (%)	8 (40)	1 (25)	10 (56)	5 (39)
FEV_1 % predicted*	66 (53-93)	74 (18-N/A)	75 (63-87)	74 (55-99)
FVC % predicted*	83 (64-99)	79 (16-N/A)	92 (80-103)	76 (56-99)
BMI z-score*	-0.36 (-0.52- -0.36)	-0.62 (-1.09- -0.42)	-0.56 (-0.75- -0.22)	-0.62 (-1.58-0.53)
No. of outpatient visits*	7 (4-10)	2 (2-3)	6 (5-9)	2 (0-6)
No. of IV antibiotic courses*	2 (1-4)	2 (0-7)	1 (0-2)	0 (0-0)
No of IV antibiotic days*	28 (14-58)	33 (0-106)	5 (0-26)	0 (0-0)
% Inhaled antibiotic†	15	25	67	8
% Dornase alpha†	35	75	50	46
% HTS†	25	0	22	54
% Azithromycin†	45	75	89	39
Antibiotic susceptibility testing methodology	CLSI	CLSI	CDS	CDS
P. aeruginosa antibiotic resistance, %				
- Aminoglycosides	50	75	39	39
- β-lactam agents	30	50	11	8
- Fluoroquinolones‡	45	50	31	0
- MARPA§	35	75	25	8

Due to the skewed distributions of the data the differences between continuous variables were examined by the Kruskal-Wallis test and reported as medians and inter-quartile ranges. Differences between categorical variables were examined by chi-squared tests.*In the 12-months prior to sample collection, for FEV_1% predicted this was the best value recorded for this period and the FVC and BMI recorded on that day; †in the 30-days prior to sample collection. N/A: Not available, FEV_1: Forced expiratory volume in 1 second, FVC: forced vital capacity, BMI: body-mass index, HTS: hypertonic saline, MARPA: multi-antibiotic resistant *P. aeruginosa*. Antibiotic susceptibility testing data unavailable on ‡14 samples and §12 samples. Site A(pX): denotes a paediatric treatment centre in State A. CDS: Continuous Dichotomous Susceptibility. CLSI: Clinical and Laboratory Standards Institute, EUCAST: European Committee on Antimicrobial Sensitivity Testing.

Table 2-3 Comparison of clinical characteristics, treatment burden and *Pseudomonas aeruginosa* resistance profiles of patients attending each Australian adult centres included in the study.

Part A. The major adult treatment centre in each state.

	Whole Cohort (n=706)	State-A(a2) (n=171)	State-B(a2) (n=108)	State-C(a3) (n=124)	State-D(a1) (n=72)	State-E(a1) (n=92)	State-F(a1) (n=18)	P-value‡
Age	27 (22-33)	26 (22-33)	27 (23-35)	29 (24-38)	27 (23-32)	27 (23-35)	27 (23-35)	**0.013**
Males, number (%)	409 (58)	101 (59)	63 (58)	70 (57)	49 (68)	48 (52)	12 (67)	0.42
FEV_1 % predicted*	53 (38-69)	51 (36-68)	59 (41-74)	47 (34-63)	57 (37-78)	49 (36-64)	49 (36-64)	**0.026**
FVC % predicted*	73 (58-86)	73 (55-87)	76 (61-94)	68 (57-83)	75 (60-91)	65 (52-77)	65 (52-77)	**<0.001**
BMI*	21 (19-24)	21 (19-25)	22 (20-25)	21 (20-24)	22 (20-23)	22 (19-25)	22 (19-25)	0.26
No. of outpatient visits*	5 (3-9)	7 (4-10)	3 (1-6)	6 (5-10)	3 (1-5)	6 (2-11)	8 (4-12)	**<0.001**
No. of IV antibiotic courses*	1 (0-2)	1 (0-3)	0 (0-1)	2 (1-3)	1 (0-3)	1 (0-2)	1 (0-2)	**<0.001**
No of IV antibiotic days*	14 (0-32)	15 (0-36)	0 (0-14)	21 (7-34)	14 (0-38)	14 (0-32)	7 (0-22)	**<0.001**
% Inhaled antibiotic†	48	23	65	65	38	52	70	**<0.001**
% Dornase alpha†	50	36	47	62	61	44	94	**<0.001**
% HTS†	32	29	38	44	33	27	53	**<0.05**
% Azithromycin†	65	47	84	72	52	55	59	**<0.001**
Antibiotic susceptibility testing methodology	-	CLSI	CLSI	CLSI	CLSI	CLSI	CDS	
P. aeruginosa antibiotic resistance, %								
- Aminoglycosides§	53	42	62	68	60	45	53	**<0.001**
- β-lactam agents¶	16	18	18	14	6	10	5	0.08
- Fluoroquinolones¥	46	27	37	79	35	37	68	**<0.001**
- MARPA¥	35	22	38	62	21	24	44	**<0.001**

Part B. Other adult treatment centres included in the study

	State A(a4)	State A(a5)	State B(a4)	State C(a3)
Participants, n (% of total clinic population)	36 (64)	15 (30)	20 (35)	50 (29)
Age	25 (21-32)	25 (18-28)	24 (21-28)	23 (18-28)
Males, number (%)	21 (58)	8 (53)	11 (55)	26 (52)
FEV_1 % predicted*	46 (37-64)	51 (37-83)	54 (42-87)	62 (49-81)
FVC % predicted*	66 (53-74)	79 (50-94)	72 (60-87)	78 (66-90)
BMI*	21 (20-24)	19 (17-21)	22 (20-24)	21 (19-24)
No. of outpatient visits*	4 (3-6)	4 (3-9)	9 (4-12)	4 (2-6)
No. of IV antibiotic courses*	1 (0-3)	3 (0-4)	1 (0-2)	1 (0-3)
No of IV antibiotic days*	13 (0-28)	64 (0-85)	14 (0-21)	15 (0-43)
% Inhaled antibiotic†	72	40	15	50
% Dornase alpha†	39	73	55	56
% HTS†	19	13	5	26
% Azithromycin†	92	80	80	72
Antibiotic susceptibility testing methodology	CLSI	CLSI	CDS	CLSI
P. aeruginosa antibiotic resistance, %				
- Aminoglycosides‡	39	73	65	42
- β-lactam agents§	40	40	20	18
- Fluoroquinolones¶	58	47	40	64
- MARPA¶	36	40	50	38

Due to the skewed distributions of the data the differences between continuous variables were examined by the Kruskal-Wallis test and reported as medians and inter-quartile ranges. Differences between categorical variables were examined by chi-squared tests. *In the 12-months prior to sample collection, for FEV_1% predicted this was the best value recorded for this period and the FVC and BMI recorded on that day; †in the 30-days prior to sample collection. FEV_1: forced expiratory volume in 1 second, FVC: forced vital capacity, BMI: body-mass index, HTS: hypertonic saline, MARPA: multi-antibiotic resistant *P. aeruginosa*. Antibiotic susceptibility testing data unavailable on ‡one, §two and ¶seven samples. State A(aX): denotes an adult treatment centre in State A. CDS: Continuous Dichotomous Susceptibility. CLSI: Clinical and Laboratory Standards Institute, EUCAST: European Committee on Antimicrobial Sensitivity Testing.

2.4.1 Paediatric centres

Eight paediatric centres (4-61 patients) from four states provided samples. Of patients culturing *P. aeruginosa*, 31% had MARPA isolates, with 43%, 21% and 30% of all isolates showing resistance to aminoglycoside, β-lactam and fluoroquinolone antibiotics respectively (Table 2.2). MARPA and resistance to β-lactam and fluoroquinolone antibiotics correlated with increased number of intravenous antibiotic days and outpatient visits in the preceding year (Table 2.4).

Comparing the largest centres in each state (Table 2.2A) showed similar patient characteristics, except for higher patient ages in State-D (p) and lower BMI z-scores in State-B (p). Large differences in intravenous antibiotic use, maintenance therapies and antibiotic resistance rates existed between states. Logistic regression demonstrated intravenous antibiotic usage and state were each independently associated with MARPA, while linear regression found recent inhaled antibiotic use, reduced FEV_1% predicted and state were associated independently with intravenous antibiotic usage (Tables 2.5 and 2.6).

2.4.2 Adult centres

Ten adult centres (15-171 patients) from six states participated. Of those harbouring *P. aeruginosa*, 35% had MARPA, with 53%, 16% and 46% showing resistance to aminoglycoside, β-lactam and fluoroquinolone antibiotics respectively (Table 2.3). In contrast with paediatric centres, the number of intravenous antibiotic courses and days did not correlate with antibiotic resistance. However, a weak correlation existed between number of outpatient visits, maintenance therapies (mucolytics and azithromycin) and MARPA isolates (Table 2.4).

Comparisons between the major centres demonstrated greater heterogeneity across clinical parameters than seen in paediatric centres. Similarly, there were significant differences in intravenous antibiotic and maintenance treatment usage, and rates of antibiotic resistance between adult centres (Table 2.3A). Treatment centre and BMI were independent determinants of MARPA. However, no relationship was found between MARPA and intravenous antibiotic administration in the previous 12-months. Being female, receiving

inhaled antibiotics, reduced FEV_1% predicted and state were independently associated with intravenous antibiotic usage (Table 2.5 and 2.6).

2.4.3 Shared *P. aeruginosa* strains

Of the 934 patients, 574 (61%) had the same unique (289), AUST-01 (160) or AUST-02 (125) genotype for each of their three isolates typed. Rates of AUST-01 (19%) and AUST-02 (14%) were significantly higher in adult than paediatric centres (9% and 8% respectively). Clinical parameters between the three groups were similar, however, compared to patients with unique isolates, AUST-01 and AUST-02 infected patients were more likely to have MARPA, β-lactam or aminoglycoside resistant isolates. Moreover, AUST-01 isolates had higher fluoroquinolone resistance rates (Table 2.7). Infection with a shared strain was an independent predictor of both increased intravenous antibiotic usage and MARPA (Tables 2.8 and 2.9).

2.4.4 Testing Methodology and MARPA

Overall, 743 (79.6%) sputum samples were collected during out-patient visits. Within each state MARPA prevalence did not differ significantly between inpatient and outpatient samples (Table 2.10) or between mixed and pure isolate selection techniques (30.4% versus 35.4% respectively, P=0.16). Although samples analysed using CDS or CLSI breakpoints provided similar MARPA rates (28.6% *versus* 30.1%, p=0.76), these were significantly higher (62.3%) in the single centre (C(a)2) employing EUCAST breakpoint values (Table 2.11).

Table 2-4 Correlation co-efficients of treatment burden with antibiotic resistance in all participating Australian paediatric and adult centres.

	Outpatient Visits	No. of IV Antibiotic Courses	No. of IV antibiotic days	Dornase alpha	Azithromycin	HTS	Inhaled Antibiotic
MARPA	0.09* / 0.15*	0.06 / 0.14*	0.04 / 0.16*	0.09* / 0.13	0.09* / 0.13	0.09* / 0.16*	0.06 / -0.11
β-Lactam Resistance	0.08 / 0.20†	0.01 / 0.20†	0.00 / 0.20†	0.04 / 0.13	0.09* / 0.07	0.03 / 0.19†	0.01 / -0.07
Aminoglycoside Resistance	0.01 / 0.04	0.03 / 0.08	0.02 / 0.09	0.09 / 0.12	0.06 / 0.14*	0.07 / 0.14*	0.01 / -0.12
Fluoroquinolone Resistance	0.05 / 0.16*	0.06 / 0.13	0.04 / 0.16*	0.07 / 0.08	0.05 / 0.16*	0.11† / 0.09	0.10* / 0.04

Spearmans Rho, Correlation significant at the *0.05 or †0.01 level (2-tailed). Paediatric centres (grey triangles), adult centres (white triangles). IV: intravenous; HTS: hypertonic saline.

Table 2-5 Factors associated with MARPA by logistic regression analysis between major treatment centres.

	Paediatric Centres (States = 4, n=150)		Adult Centres (States = 6, n=572)	
Variable	**Odds ratio (95% CI)**	**P-value**	**Odds ratio (95% CI)**	**P-value**
Univariate				
Age	1.08 (1.00-1.18)	**0.049**	1.01 (0.99-1.03)	0.26
Female gender	0.97 (0.50-1.90)	0.94	1.00 (0.71-1.42)	0.98
FEV_1% predicted*	0.35 (0.07-1.72)	0.20	0.55 (0.22-1.17)	0.11
BMI†	1.20 (0.83-1.74)	0.32	0.94 (0.90-0.99)	**0.014**
Inhaled antibiotic‡	0.50 (0.24-1.01)	0.05	1.37 (0.97-1.95)	0.08
Dornase alpha‡	2.09 (1.00-4.36)	**0.049**	1.51 (1.07-2.13)	**0.020**
HTS‡	2.86 (1.38-5.95)	**0.005**	1.71 (1.19-2.44)	**0.003**
Azithromycin‡	1.83 (0.92-3.66)	0.08	1.49 (1.06-2.14)	**0.032**
No. of IV antibiotic days§	1.02 (1.01-1.03)	**0.001**	1.00 (0.99-1.01)	0.53
States A	1.00 (ref)		1.00 (ref)	
B	0.17 (0.06-0.52)	**0.002**	2.21 (1.29-3.78)	**0.004**
C	0.09 (0.03-0.26)	**<0.001**	5.97 (3.55-10.03)	**<0.001**
D	1.64 (0.58-4.64)	0.350	0.95 (0.48-1.87)	0.88
E	-		1.14 (0.62-2.08)	0.68
F	-		3.25 (1.23-8.60)	**0.018**
Multivariate				
BMI	¶		0.93 (0.88-0.99)	**0.018**
Inhaled antibiotic	0.45 (0.18-1.13)	0.088	¶	
No. of IV antibiotic days	1.02 (1.00-1.03)	**0.021**	¶	
State A	1.00 (ref)		1.00 (ref)	
B	0.24 (0.08-0.80)	**0.020**	2.36 (1.37-4.07)	**0.002**
C	0.14 (0.05-0.41)	**<0.001**	6.12 (3.62-10.37)	**<0.001**
D	1.14 (0.35-3.70)	0.832	0.99 (0.50-1.96)	0.970
E	-		1.24 (0.67-2.28)	0.497
F	-		2.68 (0.93-7.72)	0.068

BMI: body-mass index, FEV_1: forced expiratory volume percentage, HTS: hypertonic saline, IV: intravenous. *Best FEV_1 recorded in prior 12-months, †z-scores used for paediatric data, ‡in the 30-days prior to sample collection, §in the 12-months prior to sample collection, ¶non-significant in univariate analysis and not carried forward to multivariate analysis.

Table 2-6 Factors associated with intravenous antibiotic usage by linear regression analysis among subjects treated in a major treatment centre.

	Paediatric Centres (States = 4, n=150)		**Adult Centres (States = 6, n=572)**	
Variable	**β (95% CI)**	**P-value**	**β (95% CI)**	**P-value**
Univariate				
Age	1.42 (0.23-2.61)	**0.020**	0.01 (-0.26-0.27)	0.95
Female gender	5.65 (-5.23-16.52)	0.31	12.67 (8.01 – 17.33)	**<0.001**
FEV_1% predicted*	-83.87 (-106.24- -61.50)	**<0.001**	-48.16 (-58.84 - -37.49)	**<0.001**
BMI†	-7.77 (-13.54- -1.99)	**0.009**	-1.84 (-2.49 - -1.18)	**<0.001**
Inhaled antibiotic‡	17.87 (6.83-28.91)	**0.002**	5.44 (0.60-10.27)	**0.028**
Dornase alpha‡	10.46 (-0.71-21.63)	0.07	10.08 (5.44-14.71)	**<0.001**
HTS‡	27.79 (16.07-39.50)	**<0.001**	7.53 (2.60-12.45)	**0.003**
Azithromycin‡	28.91 (18.64-39.19)	**<0.001**	11.46 (6.67-16.24)	**<0.001**
States A	1.00 (ref)		1.00 (ref)	
B	-9.47 (-24.40-5.46)	0.21	-16.93 (-23.76 - -10.10)	**<0.001**
C	-10.63 (-23.24-1.99)	0.10	0.43 (-6.14 – 7.00)	0.90
D	28.64 (12.25-45.04)	**0.001**	0.28 (-7.54 – 8.10)	0.94
E	-		-4.59 (-11.76 – 2.57)	0.21
F	-		-10.77 (-24.81 – 3.26)	0.13
Multivariate				
Female gender	§		10.24 (5.63 – 14.86)	**<0.001**
BMI	§		-0.60 (-1.28 – 0.07)	0.08
Inhaled antibiotic	10.63 (0.60-20.67)	**0.038**	5.79 (1.03 – 10.54)	**0.017**
FEV_1% predicted	-78.99 (-100.11- -57.87)	**<0.001**	-39.56 (-50.85- -28.28)	**<0.001**
State A	1.00 (ref)		1.00 (ref)	
B	-15.19 (-28.52- -1.87)	**0.026**	-17.07 (-23.75- -10.39)	**<0.001**
C	-15.62 (-27.43- -3.81)	**0.010**	-3.60 (-10.01 – 2.81)	0.27
D	26.49 (11.41-41.56)	**0.001**	3.08 (-5.43 – 11.59)	0.48
E	-		-7.34 (-14.15- -0.53)	**0.04**
F	-		-12.13 (-25.77-1.51)	0.08

BMI: body-mass index, FEV_1%: forced expiratory volume percentage, HTS: hypertonic saline, *Best FEV_1 recorded in prior 12-months, †z-scores used for paediatric data, ‡in the 30-days prior to sample collection, §non-significant in univariate analysis and not carried forward to multivariate analysis.

Table 2-7 Comparison of patient demographics and antibiotic resistance in patients with unique, AUST-01 and AUST-02 P. aeruginosa strain infection.

	Unique (n=289)	AUST-01 (n=160)	AUST-02 (n=125)
Age	24 (16-33)	24 (20-28)	25 (20-28)
FEV_1 % predicted*	60 (43-76)	54 (42-68)	56 (37-74)
FVC % predicted*	76 (60-92)	73 (62-87)	74 (55-88)
BMI*	21 (18-23)	21 (19-23)	20 (19-23)
P. aeruginosa antibiotic resistance, %			
- Aminoglycosides	39	60[§]	52[†]
- β-lactam agents	13	22[‡]	22[†]
- Fluoroquinolones	39	51[†]	40
- MARPA[¶]	27	43[§]	37[†]

Due to the skewed distributions of the data the differences between continuous variables were examined by Kruskal-Wallis test and reported as median and inter-quartile range, and 2x2 tables and chi-squared tests were used to compare dichotomous variables for AUST-01 and AUST-02 to unique strains. *For FEV_1% predicted this was the best value recorded for the 12-months prior to sample collection and the FVC and BMI recorded on that day. [†]P<0.05, [‡]P<0.01, [§]P<0.001. FEV_1: forced expiratory volume in 1 second, FVC: forced vital capacity, BMI: body-mass index, MARPA: multi-antibiotic resistant *P. aeruginosa*. [¶]Antibiotic susceptibility testing unavailable on 14 samples.

Table 2-8 Factors associated with MARPA by logistic regression analysis among subjects infected with unique, AUST-01 or AUST0-2 treated in a major treatment centre.

	Paediatric Centres (States = 4, n=100)		**Adult Centres (States= 6, n=353)**	
Variable	**Odds ratio (95% CI)**	**P-value**	**Odds ratio (95% CI)**	**P-value**
Univariate				
Age	1.08 (1.00-1.18)	**0.049**	1.01 (0.99-1.03)	0.26
Female gender	0.97 (0.50-1.90)	0.94	1.00 (0.71-1.42)	0.98
FEV_1% predicted*	0.35 (0.07-1.72)	0.20	0.55 (0.22-1.17)	0.11
BMI†	1.20 (0.83-1.74)	0.32	0.94 (0.90-0.99)	**0.014**
Inhaled antibiotic‡	0.50 (0.24-1.01)	0.052	1.37 (0.97-1.95)	0.08
Dornase alpha‡	2.09 (1.00-4.36)	**0.049**	1.51 (1.07 – 2.13)	**0.020**
HTS‡	2.86 (1.38-5.95)	**0.005**	1.71 (1.19-2.44)	**0.003**
Azithromycin‡	1.83 (0.92-3.66)	0.08	1.49 (1.06-2.14)	**0.032**
No. of IV antibiotic days§	1.02 (1.01-1.03)	**0.001**	1.00 (0.99-1.01)	0.53
Unique *P. aeruginosa*	1.00 (ref)		1.00 (ref)	
AUST-1	3.96 (1.06-14.75)	**0.040**	2.20 (1.31-3.69)	**0.003**
AUST-2	19.17 (5.14-71.41)	**<0.001**	1.24 (0.71-2.18)	0.44
States A	1.00 (ref)		1.00 (ref)	
B	0.17 (0.06-0.52)	**0.002**	2.21 (1.29-3.78)	**0.004**
C	0.09 (0.03-0.26)	**<0.001**	5.97 (3.55-10.03)	**<0.001**
D	1.64 (0.58-4.64)	0.35	0.95 (0.48-1.87)	0.88
E	-		1.14 (0.62-2.08)	0.68
F	-		3.25 (1.23-8.60)	**0.018**
Multivariate				
State A	1.00 (ref)		1.00 (ref)	
B	0.05 (0.01-0.53)	**0.014**	3.01 (1.48-6.16)	**0.002**
C	0.05 (0.01-0.34)	**0.002**	5.82 (2.74-12.37)	**<0.001**
D	1.02 (0.13-7.70)	0.99	1.26 (0.48-3.29)	0.63
E	-		0.99 (0.46-2.12)	0.98
F	-		4.48 (0.99-20.28)	0.05
Unique *P. aeruginosa*	1.00 (ref)		1.00 (ref)	
AUST-01	5.95 (1.11-31.80)	**0.037**	1.64 (0.94-2.89)	0.08
AUST-02	4.22 (0.78-22.78)	0.09	2.36 (1.22-4.55)	**0.010**

*For FEV_1% predicted this was the best value recorded for the 12-months prior to sample collection and the FVC and BMI recorded on that day, †z-scores used for paediatric data, ‡in the 30-days prior to sample collection, §in the 12-months prior to sample collection. BMI: body-mass index, FEV1% predicted: forced expiratory volume percentage predicted, HTS: hypertonic saline, IV: intravenous,

Table 2-9 Factors associated with intravenous antibiotic usage by linear regression analysis among subjects infected with unique, AUST-01 or AUST-02 treated in a major treatment centre.

	Paediatric Centres (States = 4, n=100)		Adult Centres (States= 6, n=353)	
Variable	**β (95% CI)**	**P-value**	**β (95% CI)**	**P-value**
Univariate				
Age	1.42 (0.23 – 2.61)	**0.020**	0.01 (-0.26-0.27)	0.95
Female gender	5.65 (-5.23-16.52)	0.31	12.67 (8.01-17.33)	**<0.001**
FEV_1% predicted*	-83.87 (-106.24 - -61.50)	**<0.001**	-48.16 (-58.84- -37.49)	**<0.001**
BMI†	-7.77 (-13.54 - -1.99)	**0.009**	-1.84 (-2.49- -1.18)	**<0.001**
Inhaled antibiotic‡	17.87 (6.83 – 28.91)	**0.002**	5.44 (0.60-10.27)	**0.028**
Dornase alpha‡	10.46 (-0.71-21.63)	0.07	10.08 (5.44-14.71)	**<0.001**
HTS‡	27.79 (16.07-39.50)	**<0.001**	7.53 (2.60-12.45)	**0.003**
Azithromycin‡	28.91 (18.64-39.19)	**<0.001**	11.46 (6.67-16.24)	**0.003**
Unique *P. aeruginosa*	1.00 (ref)		1.00 (ref)	
AUST-01	21.42 (6.03-36.81)	**0.007**	10.58 (3.14-18.00)	**0.005**
AUST-02	11.85 (-3.53-27.24)	0.13	10.12 (2.34-17.91)	**0.011**
States A	1.00 (ref)		1.00 (ref)	
B	-9.47 (-24.40 – 5.46)	0.21	-16.93 (-23.76- -10.10)	**<0.001**
C	-10.63 (-23.24 – 1.99)	0.10	0.43 (-6.14-7.00)	0.90
D	28.64 (12.25 – 45.04)	**0.001**	0.28 (-7.54-8.10)	0.94
E	-		-4.59 (-11.76-2.57)	0.21
F	-		-10.77 (-24.81-3.26)	0.13
Multivariate				
Female gender	§		13.77 (7.85-19.70)	**<0.001**
FEV_1% Predicted	-57.06 (-81.23- -32.88)	**<0.001**	-38.35 (-52.21- -24.49)	**<0.001**
State A	1.00 (ref)		1.00 (ref)	
B	1.61 (-17.81-21.05)	0.87	-10.87 (-19.52- -2.21)	**0.014**
C	-3.52 (-20.91-13.85)	0.69	2.89 (-6.25-12.02)	0.54
D	22.80 (-0.93-46.52)	0.06	7.43 (-3.69-18.56)	0.19
E	-		-1.30 (-9.98-7.37)	0.77
F	-		-5.07 (-23.03-16.17)	0.64
Unique *P. aeruginosa*	1.00 (ref)		1.00 (ref)	
AUST-01	22.63 (7.71-37.55)	**0.003**	11.10 (4.00-18.21)	**0.002**
AUST-02	9.16 (-8.79-27.12)	0.31	9.08 (1.38-16.79)	**0.021**

*For FEV_1% predicted this was the best value recorded for the 12-months prior to sample collection and the FVC and BMI recorded on that day, †z-scores used for paediatric data, ‡in the 30-days prior to sample collection, §non-significant in univariate analysis and not carried forward to multivariate analysis. BMI: body-mass index, FEV1% predicted: forced expiratory volume percentage predicted, HTS: hypertonic saline.

Table 2-10 Relationship between sampling location and rates of MARPA.

Site	Number of samples collected as an inpatient (% MARPA infected)	Number of samples collected as an outpatient (% MARPA infected)	P-value
A(p1)	10 (70)	37 (51.4)	0.48
A(a2)	26 (15.4)	140 (22.9)	0.40
A(p3)	2 (50.0)	18 (33.3)	1.00
A(a4)	6 (50.0)	30 (33.3)	0.65
A(a5)	3 (33.3)	12 (41.7)	1.00
A(p6)	1 (100.0)	3 (66.7)	1.00
B(p1)	0	16 (25.0)	n/a
B(a2)	3 (66.7)	105 (37.1)	0.56
B(p3)	4 (0)	27 (18.5)	1.0
B(a4)	3 (66.7)	17 (47.1)	1.0
B(p5)	0	13 (7.7)	n/a
C(p1)	16 (12.5)	46 (8.7)	0.64
C(a2)	78 (61.5)	44 (63.6)	0.82
C(a3)	6 (50)	44 (36.4)	0.66
D(a1)	2 (50.0)	70 (20.0)	0.38
D(p2)	14 (71.4)	9 (55.6)	0.66
E(a1)	5 (40.0)	87 (23.0)	0.59
F(a1)	5 (40.0)	12 (41.7)	1.00

n/a. Not applicable Site A(a/pX): denotes a treatment centre in State A (adult/paediatric). MARPA: Multi-antibiotic resistant *P. aeruginosa*.

Table 2-11 Rate of MARPA stratified by testing methodology.

Sampling Methodology	No. of sample (No. of centres)	Percentage MARPA
Isolate Selection		
- Mixed	210 (6)	30.0%
- Pure	724 (12)	35.4%*
Reference Range		
- CLSI	695 (12)	30.1%
- CDS	98 (5)	28.6%†
- EUCAST	122 (1)	62.3%‡

Pure: isolate selected from the plate for sensitivity testing, Mixed: sweep taken from the plate for sensitivity testing, CDS: Continuous Dichotomous Susceptibility; CLSI: Clinical and Laboratory Standards Institute; EUCAST: European Committee on Antimicrobial Sensitivity Testing. *P=0.16 compared to sweep, †P=0.76 compared to CLSI, ‡P<0.001 compared to either CLSI or CDS.

2.5 Discussion

By linking data from the ACFDR and the national ACPinCF study we found that Australian CF patients harbouring *P. aeruginosa* had MARPA rates, intravenous antibiotic usage and maintenance treatment practices that varied noticeably between centres. In children, antibiotic treatment intensity correlated with antibiotic resistance, while intravenous antibiotic treatments were associated with recent inhaled antibiotic use, reduced lung function and treating centre. In contrast, in adults MARPA was associated with centre and BMI, but not with intravenous antibiotic treatment intensity, while being female, recent inhaled antibiotics, reduced lung function and treating centre were also independently associated with intravenous antibiotic usage.

Inhaled tobramycin, frequent intravenous antibiotics, CF-related diabetes, hospitalisation for acute exacerbations, and treatment in a centre with an already high MARPA prevalence were identified previously as risk factors for MARPA in CF patients with newly acquired *P. aeruginosa* infection.[4] Longitudinal studies of *P. aeruginosa* infection in non-CF individuals

have also found MARPA prevalence closely parallels intravenous antibiotic use.[234, 235] Thus our observations that MARPA in CF adults did not correlate with intravenous antibiotic usage or maintenance treatment burden in our study was unexpected. This may indicate more complex factors influence *P. aeruginosa* phenotype in these older patients, including a greater impact from shared strains, antibiotic resistance related to long-term, cumulative antibiotic exposure from childhood and the altered microenvironment present in more severe bronchiectasis.(213, 236) For example, despite similar lung function and clinicians being unaware of strain typing status, patients with highly-prevalent AUST-01 and AUST-02 strains were more likely to receive greater intensity of antibiotic treatments and to possess MARPA isolates than those infected with unique strains.

It is important to highlight that in chronic *P. aeruginosa* infection antibiotic susceptibility does not predict clinical response to treatment and the clinical significance of MARPA in CF remains contentious.[210, 237] While a single centre study involving CF adults reported an increased rate of pulmonary decline in patients with MARPA infection,[238] this was not supported by a recent large data registry-based study, which found instead MARPA were more likely markers for advanced disease or intensive antibiotic therapy.[239] Furthermore, a CF Foundation Benchmarking Project, identified early and aggressive antibiotic management of pulmonary decline, to be a distinguishing feature of CF centres (paediatric and adult) achieving outstanding clinical outcomes.[203]

The prevalence of MARPA among paediatric centres is comparable with the rates reported in an earlier single centre study and are concerning.(240) However, in two large paediatric centres providing the most intense treatments there was some evidence to suggest improved nutritional outcomes despite associated higher antibiotic resistance, while there was no clear association between MARPA and adverse clinical outcomes. Nevertheless, increasing treatment and its complexity in response to MARPA infection may lead to treatment-related morbidity and promote emergence of shared strains.[5, 241, 242],[243]

These findings underline the dilemma facing CF physicians. Maintaining lung function and quality of life, while attempting to minimise antibiotic resistance and treatment-related adverse effects is especially challenging when the mechanisms of antibiotic action in CF lung disease are incompletely understood(244). Although consensus recommendations for managing pulmonary exacerbations and prescribing maintenance therapies have been

published, their uptake may be variable.[127, 202, 245] Recognised barriers to adopting guidelines into clinical practice include: i) lack of knowledge or agreement with recommendations, ii) inertia in adopting change to management and iii) external barriers (e.g. drug availability and cost, as well as resistance to adopting specific therapies) and may have contributed to their incomplete uptake and variations in practice seen in Australian CF centres.[246] A US-based survey of CF physicians identified perceived benefit and presence of external barriers to be major factors influencing their willingness to adopt guidelines.[247]

Our study has several limitations. First, *in-vitro* antibiotic susceptibility testing methods varied between CF centres (Table 2.1), however there are no standardised approaches to antibiotic resistance testing nationally or internationally.(248) The large number of isolates received by the reference laboratory meant it was not feasible to confirm the susceptibility of each isolate using a standard method. However, antibiotic resistance rates did not differ significantly between mixed versus pure testing methodologies. Furthermore, despite CLSI applying higher minimum inhibitory concentration (MIC) breakpoints for determining resistance to b-lactams antibiotics than CDS (Table 2.12), centres applying CLSI methodology showed higher rates generally of antibiotic resistance (Table 2.2 and 2.3). These data indicate that differences between CDS and CLSI testing methodologies are unlikely to have contributed to our findings. MARPA rates were however higher in the single centre using EUCAST MIC breakpoints, but we were unable to determine if this was from testing methodology or genuinely higher resistance rates. Second, our cohort included only those capable of providing sputum. This meant that those with more severe lung disease were likely to be selected, as younger patients and those with mild disease often cannot produce a sputum sample on demand.[249, 250] Third, as this study was cross-sectional, we could not differentiate between those with chronic or intermittent infections. This is especially relevant in young children where newly infecting strains are more likely to be susceptible to anti-pseudomonal antibiotics. However, paediatric subjects were on average 6-years older than the mean age of the clinic population from where they were selected (data not shown) and had relatively high rates of antibiotic resistance, suggesting that most were chronically infected. Fourth, the cross-sectional design also meant that we could not ascertain if increased antibiotic resistance in adults was 'imported' from a paediatric centre with high antibiotic use or resulted from a lifetime of accumulated antibiotic exposure and in addition whether MARPA isolates were associated directly with worse clinical outcomes. Fifth, we did not collect information on the type of intravenous antibiotics prescribed for each course treatment, which may have varied

between centres and influenced induction of antibiotic-resistant strains.[243] Finally, maintenance therapies were recorded from hospital dispensing data systems and patient medical records, which may not represent actual treatment adherence.

Table 2-12 Equivalent susceptible minimum inhibitory concentration breakpoints of the disk diffusion methodologies.

	Minimal inhibitory concentrations (mg/L)		
Antibiotic	CLSI	CDS	EUCAST
Gentamicin	≤4	≤4	≤4
Tobramycin	≤4	≤4	≤4
Amikacin	≤16	≤16	≤8
Ceftazidime	≤8	≤4	≤8
Cefepime	≤8	≤2	≤8
Ticarcillin-clavulanate	≤64/2	≤16/2	≤16
Piperacillin	≤64	≤16	≤16
Piperacillin-tazobactam	≤64/4	≤16/2	≤16
Aztreonam	≤8	≤8	≤1
Meropenem	≤4	≤2	≤2
Imipenem	≤4	≤4	≤4
Ciprofloxacin	≤1	≤1	≤0.5
Colistin sulphate	≤2	NA	≤2

CLSI: Clinical and Laboratory Standards Institute, CDS: Continuous Dichotomous Susceptibility, EUCAST: European Committee on Antimicrobial Sensitivity Testing. mg/L: milligrams per litre.

2.6 Conclusions.

This national study found large between-centre differences in treatment practices and *P. aeruginosa* antibiotic resistance profiles. Increased antibiotic resistance in paediatric, but not adult, centres was associated with intravenous antibiotic usage, while in both paediatric and adult centres MARPA was not associated with worse lung function. We plan to continue monitoring this cohort longitudinally to determine whether MARPA isolates do actually result in worse clinical outcomes or simply reflect more advanced disease and/or intensive treatment strategies adopted by some centres. This study emphasises the need to develop a greater understanding of how antibiotics work in CF lung disease so that they can be used most effectively and safely. While awaiting these developments greater communication between centres may allow standardised national therapeutic guidelines to be developed to

promote the pulmonary benefits of early intensive treatment, while reducing the risk of treatment-related morbidity.

Chapter 3. Response of the CF lung Microbiome to intravenous antibiotics .

3.1 Abstract.

Introduction: Chronic airway infection in adults with CF is polymicrobial and the impact of intravenous (IV) antibiotics on the bacterial community composition is poorly understood. We employed culture-independent molecular techniques to explore the early effects of IV antibiotics on the CF airway microbiome.

Methods: DNA was extracted from sputum samples collected from adult subjects with CF at three time points (before commencement of treatment, and at day 3 and day 8-10 of IV antibiotics) during the treatment of an infective pulmonary exacerbation. Microbial community profiles were derived through analysis of bacterial-derived 16S ribosomal RNA by pyrosequencing and changes over time compared.

Results: Fifty nine sputum samples were collected during 24 pulmonary exacerbations from 23 subjects. Between treatment onset and day 3 there was a significant reduction in the relative abundance of *Pseudomonas* and increased microbial diversity. By day 8-10, bacterial community composition was similar to pre-treatment. Changes in community composition did not predict improvements in lung function.

Conclusion: The relative abundance of *Pseudomonas* falls rapidly in subjects with CF receiving IV antibiotic treatment for a pulmonary exacerbation and is accompanied by an increase in overall microbial diversity. However, this effect is not maintained beyond the first week of treatment.

3.2 Introduction

Despite advances in the management of cystic fibrosis (CF), chronic pulmonary infection remains responsible for most patient morbidity and mortality(1). Culture-dependent analysis of CF airway infection reveals that *Pseudomonas aeruginosa* (*P. aeruginosa*) is the dominant bacterial pathogen in most adults with CF(251). However, the recent application of culture-independent molecular techniques, based on the sequencing of the gene encoding bacterial 16S ribosomal RNA (rRNA), have revealed a complex microbiome in the CF airway(79, 80, 136, 252-255).

Chronic infection with *P. aeruginosa* is associated with an increased rate of lung function decline, increased frequency of pulmonary exacerbations, impaired quality of life and increased mortality(200, 201). Guidelines recommend that pulmonary exacerbations are treated aggressively with a combination of at least two intravenous (IV) anti-pseudomonal antibiotics for fourteen days(202, 256). However, this practice is based on limited evidence(256).

Two studies - one prospective and one large registry-based retrospective - have suggested that the maximum clinical and metabolic response to IV antibiotics occurs in the first week of treatment, with little additional benefit being achieved from extending treatment beyond this point(134, 135). Unfortunately, these studies did not assess the relationship between clinical improvement and microbiological response and their findings have not been considered sufficiently robust to change clinical practice.

In this current study, we utilised culture-independent molecular techniques to explore the effect on the CF airway microbiome of IV antibiotic therapy administered for the treatment of an acute pulmonary exacerbation. The focus was primarily on the impact of antibiotic therapy on microbial community composition during the first week of treatment.

3.3 Methods

3.3.1 Participants

Adult subjects (n = 23; aged 18–54 years) with CF, admitted to hospital for IV antibiotic treatment of an acute, infective pulmonary exacerbation were recruited from The Prince Charles Hospital in Queensland (n = 14) and the Royal Hobart Hospital in Tasmania (n = 9), Australia. Institutional human research and ethics committee approval was gained at both sites (HREC2008:2885 and H0009813 respectively). Individuals who had undergone lung transplantation or were using systemic immunosuppression were excluded. Based on standard microbiological cultures, all patients were infected with *Pseudomonas aeruginosa* (Appendix 2.1).

Spontaneously expectorated sputum samples were collected at day 1, before IV antibiotics (Time-point 1, TP-1), and at day 3-4 (TP-2) and day 8-10 (TP-3) following commencement of IV antibiotics.

A pulmonary exacerbation was defined clinically, based on the attending physician's assessment that the subject required intravenous antibiotics to treat an increase in respiratory symptoms or a decline in lung function.

Participant demographics and lung function and antibiotic treatment are outlined in Table 3.1. Disease severity was determined based on the participants best FEV_1 in the 12 months prior to recruitment (FEV_1 >70% mild, 40-70% moderate, <40% severe).

Table 3-1 Participant demographics, CF genotypes and antibiotic treatment[#].

Gender (male:female)	17:6
Age	27 years (18-54)
BMI	20 kg m^{-1} (17-26)
FEV_1	1.7 Litres (0.9-4.2)
FEV_1 % Predicted	50 % (21-97)
Absolute improvement in FEV_1	0.2 Litres (0-1.2)
Relative improvement in FEV_1	16 % (0-76)
Genotype[#]:	
- p.F508del homozygote	12
- p.F508del heterozygote	8
- G551D heterozygote	1
- Other / Other	2
Intravenous antibiotic treatment*:	
- β–Lactam + Aminoglycoside[$]	18
- Dual β–Lactams	2
- Dual β–Lactams + Aminoglycoside	1
- Dual β–Lactams + Colistin	1
- β–Lactam + Monobactam + Aminoglycoside	1
Azithromycin*[&]:	18

BMI: Body mass index; FEV_1: Forced expiratory volume in one second: kg: kilograms; m: metres. Age, BMI and lung function parameters are expressed as median (range).

[*] Maintenance usage of oral azithromycin, dosage either 250mg daily or 500mg alternate day.

[&] Data were unavailable for one subject.

[$] Nebulised aminoglycoside was used in one subject.

Individual participant data are available in Appendices 2.1 and 2.2.

3.3.2 Sputum collection.

Sputum was expectorated directly into 10 mL of RNA Later® (Life Technologies, Victoria) and stored for at least 24 hours at 4°C to allow full penetration into the sputum sample. Sputum samples were stored at -80°C for later batch DNA extraction.

3.3.3 DNA extraction.

A saliva-free aliquot of each frozen sputum sample was selected and manually homogenised with 500 µL lysis buffer (50 mM Tris-HCl (Sigma-Aldrich, Missouri), pH 6.8, 50 mM ethylenediaminetetraacetic acid (AnalaR, Pennsylvania), 50 mM sucrose (AnalaR), 100 mM sodium chloride (Univar, NSW) and 1% sodium dodecyl sulfate (SDS) (Amresco, Ohio). The samples were incubated at 37°C for 1 hour with 100 µL chicken egg lysozyme (100 mg/mL, Sigma-Aldrich), then at 56°C with shaking overnight with 100 µL of proteinase K (20 mg/mL, Promega, NSW) and 75 µL of 10% SDS. If samples were not completely digested, overnight incubation with additional proteinase was repeated until complete digestion was achieved. DNA was extracted and purified using PureLink Genomic DNA Mini Kit (Life Technologies, Victoria) and eluted in 50 µL kit elution buffer. Purified genomic DNA was analysed for quality (A_{260}/A_{280} ratio of 1.8 – 2.0) and concentration using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Denver) and samples were diluted to 20 ng/µL prior to DNA amplification and sequencing.

3.3.4 DNA amplification, sequencing and statistical analysis.

Sequencing was performed by Research and Testing Laboratories (Lubbock, Texas) applying Molecular Research DNA© protocols (Shallowater, Texas). The 16S rRNA gene was amplified using primers 939F (5'-TTGACGGGGGCCCGCAC-3') and 1492R (5'-TACCTTGTTACGACTT-3') and products sequenced using bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) as described previously (257). 16S rDNA amplicon sequences were processed with QIIME 1.5 (258). Sequences with ambiguous base calls ("quality score" < 25 and ≥ 6 homopolymers) were discarded. Forward and reverse primer sequences were removed allowing one mismatch. Chimeras were removed with

ChimeraSlayer (259) using default parameters. Samples with less than 500 sequence reads were excluded. 16S rDNA sequences were clustered into Operational taxonomic units (OTUs) using UCLUST v5.2.32(260), employing an identity threshold of 97%. RDP Classifier v2.2 was retrained on the database Greengenes version 13.8 and used for the taxonomic assignment of representative sequences of each OTU with a confidence threshold of 0.6(261). Genera were categorised as aerobic or anaerobic, as described previously(262). Data-mining, statistical analysis and data visualization were carried out using the Calypso software, bioinfo.qimr.edu.au/calypso, and Krona(263).

Groups were compared using paired t-tests: TP-1 *versus* TP-2; TP-1 *versus* TP-3; and TP2 *versus* TP-3. Analysis was limited to subjects for whom samples at both compared time points were available. P values were adjusted for multiple testing using the False Discovery Rate (FDR). Microbial community diversity was assessed by Shannon index (OTU level). Canonical Correlation Analysis (CCA) and Anosim were performed on the sputum microbiome at TP-1 using the relative genera abundance (number of 16S sequences assigned to each genus divided by the total number of sequences obtained for each sample). Associations between clinical variables (age and FEV_1) and the relative abundance of each genus at TP-1 or TP-3 were determined by the Pearson product-moment correlation coefficient. Analogously, Pearson correlation was utilised to infer associations between the relative abundance of genera, age and relative improvement in FEV_1 between early and late treatment. For the purpose of determining the differences between the relative abundances of bacterial groups at the genus level, Shannon diversity and FEV_1 at TP-1 and TP-3 were calculated, and then evaluated for relationships by means of Pearson correlation.

3.3.5 Real-time PCR quantification of *P. aeruginosa*.

In 18 sputum samples, from nine subjects, *P. aeruginosa* concentration was determined by RT-qPCR, using a previously established primer pair for Pseudomonas 16S RNA, *psd7*,
Forward (CAAAACTACTGAGCTAGAGTACG)
Reverse (TAAGATCTCAAGGATCCCAACGGCT)(264).

The full methods employed have previously been described(265). In brief a 0.01 to 0.15g aliquot of saliva free RNA Later® stored sputum, 1.4 ml of Tri Reagent® (Sigma-Aldrich,

Australia) and 1mL of 0.1 mm zirconia/silica beads (Biospec products inc.) were combined in a 2 mL tube and homogenised for 4 minutes at low speed using a Mini-Beadbeater (Biospec products inc.). RNA was extracted using Tri Reagent® as per the manufacturer instructions. Air-dried RNA was resuspended in 50 μL of pH 7.0 TE buffer with heating for 10 min at 55ºC. DNA was removed from the RNA using Turbo DNase (Ambion) according to the manufacturer's instructions. RNA was quantified using the Quant-iT™ RNA Assay Kit (Invitrogen) and a fluorimeter (Spectramax M2, Molecular Devices, California). RNA integrity was confirmed by agarose gel electrophoresis. For standard curve generation, RNA was serially diluted in pH 7.0 TE buffer prior to reverse transcription. cDNA was generated (20μL reactions) with SuperScript® III First-Strand Synthesis SuperMix (Invitrogen) kit, by the manufacturers methods, using a mixture of random hexamers and oligo(dT)20 for priming. RNA was removed with 1μL of the kit supplied RNAase. Each 20μL of cDNA was diluted with 46.7 μL of water before qPCR. Duplicate real-time PCR reactions were set up with 10μM of each primer according to the Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen) kit instructions, using a CAS-1200 liquid pipetting robot (Corbett Life Science, Australia). Product amplification was carried out using the following conditions, enzyme activation (2 min at 50°C), initial denaturation (2 min at 95°C), thermocyling (40 cycles of melting at 15 s at 95°C, annealed at 30 s and extended 30 s at 72°C). Data were collected at 72 °C. The annealing temperatures used was 62°C, Melt curve analysis was under-taken to confirm the specificity of the PCR products by heating from 70 to 95 °C at a linear rate of 0.5 °C/s. Excitation wavelength was 470 nm, with detection wavelength set at 510 nm. A calibrator and no-template control were included in each assay for each primer set. The calibrator consisted of CF sputum cDNA that showed expression of *P. aeruginosa* 16S rRNA. An aliquot of all RNA from sputum samples was analysed by qPCR, without prior reverse transcription, to confirm the absence of contaminating gDNA using the primer set for *P. aeruginosa* 16S rRNA. Presence of transcripts in sputum samples was defined as detection in technical replicate qPCRs of the same cDNA sample and appropriate Ct and melt analysis. Only Ct values between 6 and 31 were accepted for analysis. If a product amplified in the RNA-only control, indicating gDNA contamination, within 3 cycles of the cDNA product or with a Ct of <30 then the sample was excluded from analysis.

Pearson's correlation was used to explore the relationship between *P. aeruginosa* load and relative abundance of *Pseudomonas* at the genus level.

3.4 Results.

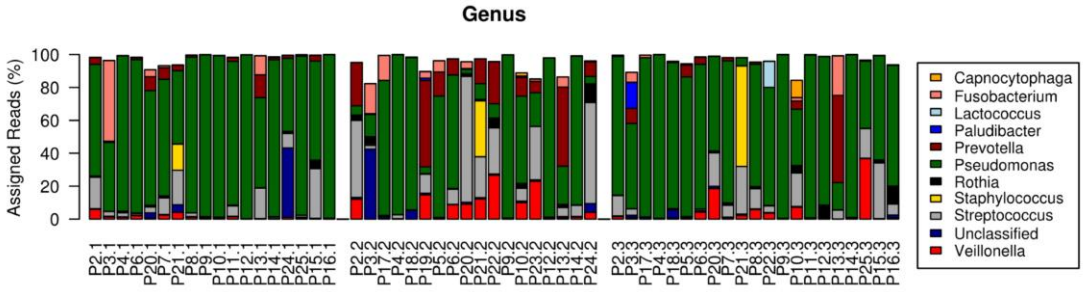
3.4.1 Sample quality

Fifty nine sputum samples were collected during 24 pulmonary exacerbations from 23 subjects. Sequencing yielded 420,145 reads with an average read length of 457 base-pairs. After quality control and chimera detection, 57 samples (18 for TP-1, 18 for TP-2 and 21 for TP-3) and 336,973 high-quality sequencing reads remained, with a median of 5478 sequences per sample and a range from 1,468 to14,164.

3.4.2 Sputum microbiota before antibiotic treatment (TP-1)

Eighteen sputum specimens were analysed at TP-1 and showed a complex microbiota, with a median of 113 OTUs per sample (range 23-210 per sample). A total of 100 different genera were observed (median, 13 per sample, range 2-39) (Figure 3.1). *Pseudomonas* was the dominant genus in 94% (17/18) of samples, with a mean relative abundance of 78.5% (range 41.9-99.6%). In one subject, *Fusobacterium* was the dominant genus (49% of 16S sequences), followed by *Pseudomonas* (41.9% of sequences). *Streptococcus* was the second most abundant genus in 13 sputum samples, with a mean relative abundance of 7.3%. The most prevalent anaerobic genera were *Prevotella* (mean relative abundance 2.7%, range 0-13.8%) and *Veillonella* (mean relative abundance 1.2%, range 0-5.9%), which were present in 72% and 78% of the subjects, respectively. Other pathogenic genera were recovered infrequently, including *Actinomyces* (44% of samples), *Staphylococcus* (17%) and *Haemophilus* (11%). The mean percentage of sequences that could not be assigned to any known genus was 3% (Appendix 2.3). Statistical analysis of the taxonomic profiles at TP-1 did not identify a relationship between global microbial community composition (rank genus) and age (CCA p=0.55; Anosim p=0.59) or FEV_1 (CCA p=0.99; Anosim p=0.7).

Figure 3-1 Taxonomic profile of all sputum samples segregated by time -point.



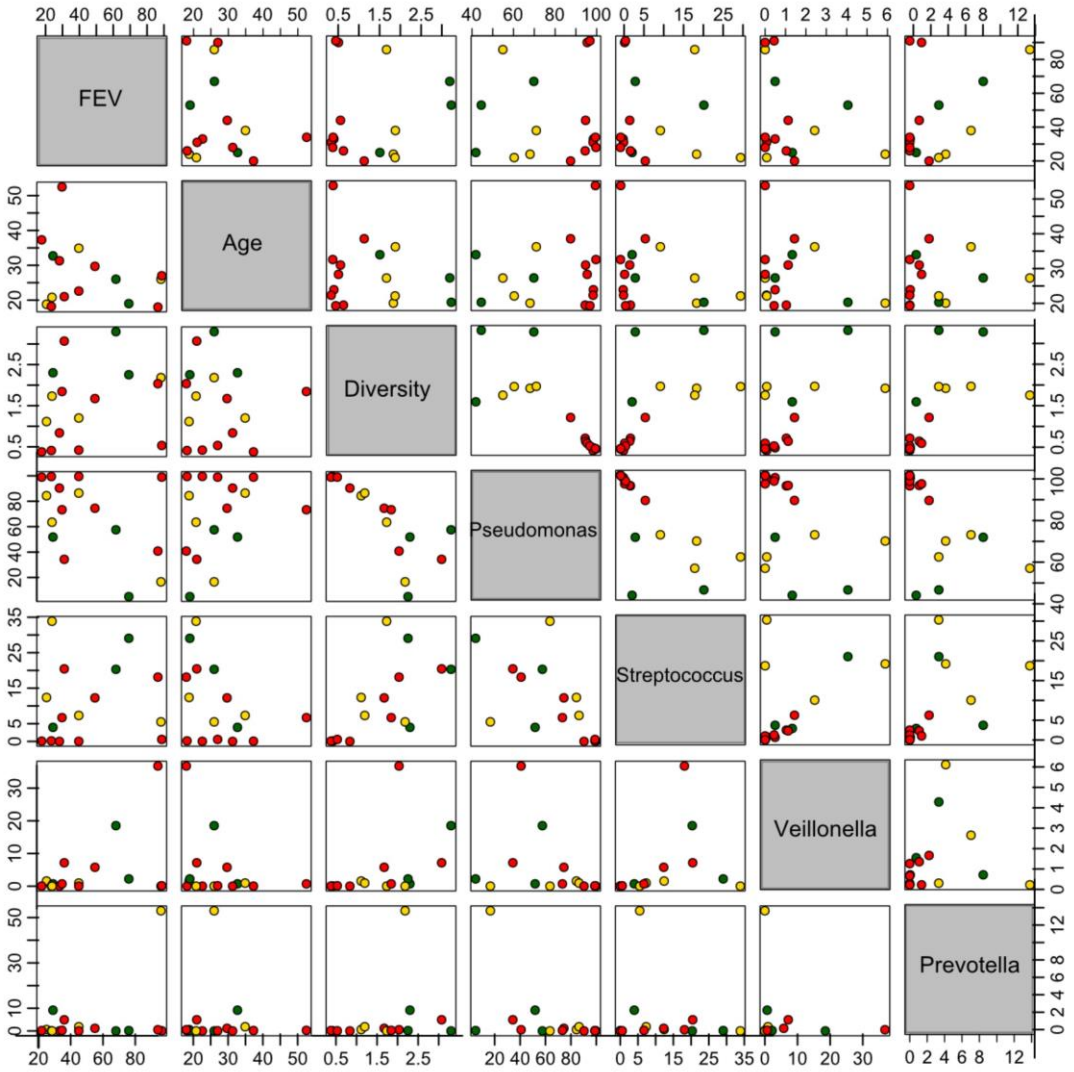
Px.y (Patient number.Timepoint). Representation limited to genera which were present in at least one sample at a relative abundance of ≥ 10% are presented.

The relative abundance of *Pseudomonas* showed a strong negative correlation with microbial diversity (Shannon index; r=-0.81, p<0.01, Pearson correlation). Conversely, the relative abundance of *Streptococcus* (r=0.63, p<0.01) and *Prevotella* (r=0.63, p<0.01) were positively correlated with community diversity (Figure 3.2). We did not observe an association between microbial diversity and subject age (p=0.29) or lung function (FEV_1, p=0.3).

3.4.3 Effects of antibiotic treatment on the airway microbiome

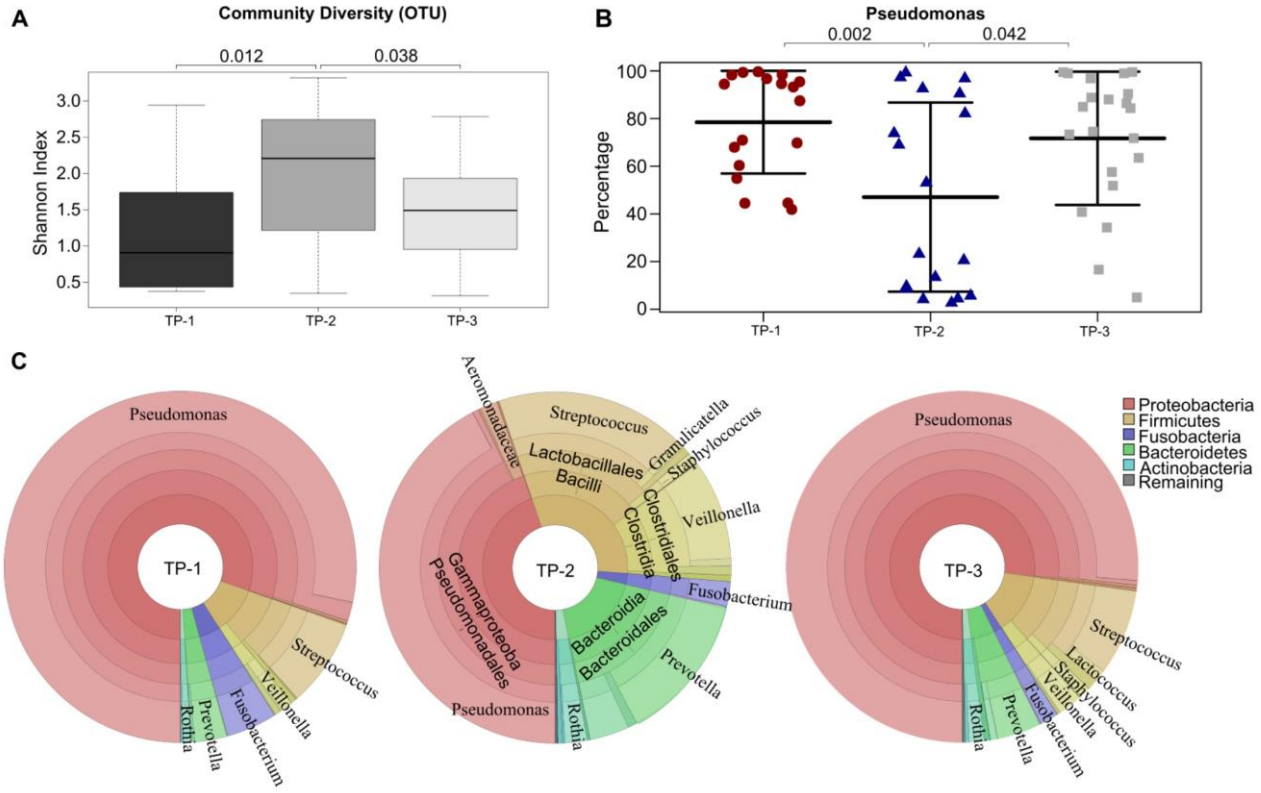
There was a significant reduction in the relative abundance of *Pseudomonas* during the first 72 hours of antibiotic treatment (p<0.005; FDR=0.02, paired t-test TP-1 vs. TP-2). The mean relative abundance of *Pseudomonas* decreased from 78.5% of 16S sequences before antibiotic treatment to 47% after 72h of treatment. The reduction in *Pseudomonas* was accompanied by a significant increase in overall microbial diversity (Shannon index; paired t-test p=0.012) and a trend towards an increase in the relative abundance of anaerobes, which was mainly driven by increases in the abundance of *Prevotella* (TP-1 2.7% of 16S sequences; TP-2 12.2%; p=0.06; FDR=0.13) and *Veillonella* (TP-1 1.2%; TP-2 6.8%; p<0.02; FDR=0.07) (Figure 3.3).

Figure 3-2 Scatterplot matrix visualizing pairwise correlations between bacterial community diversity and composition, as well as subject demographics.



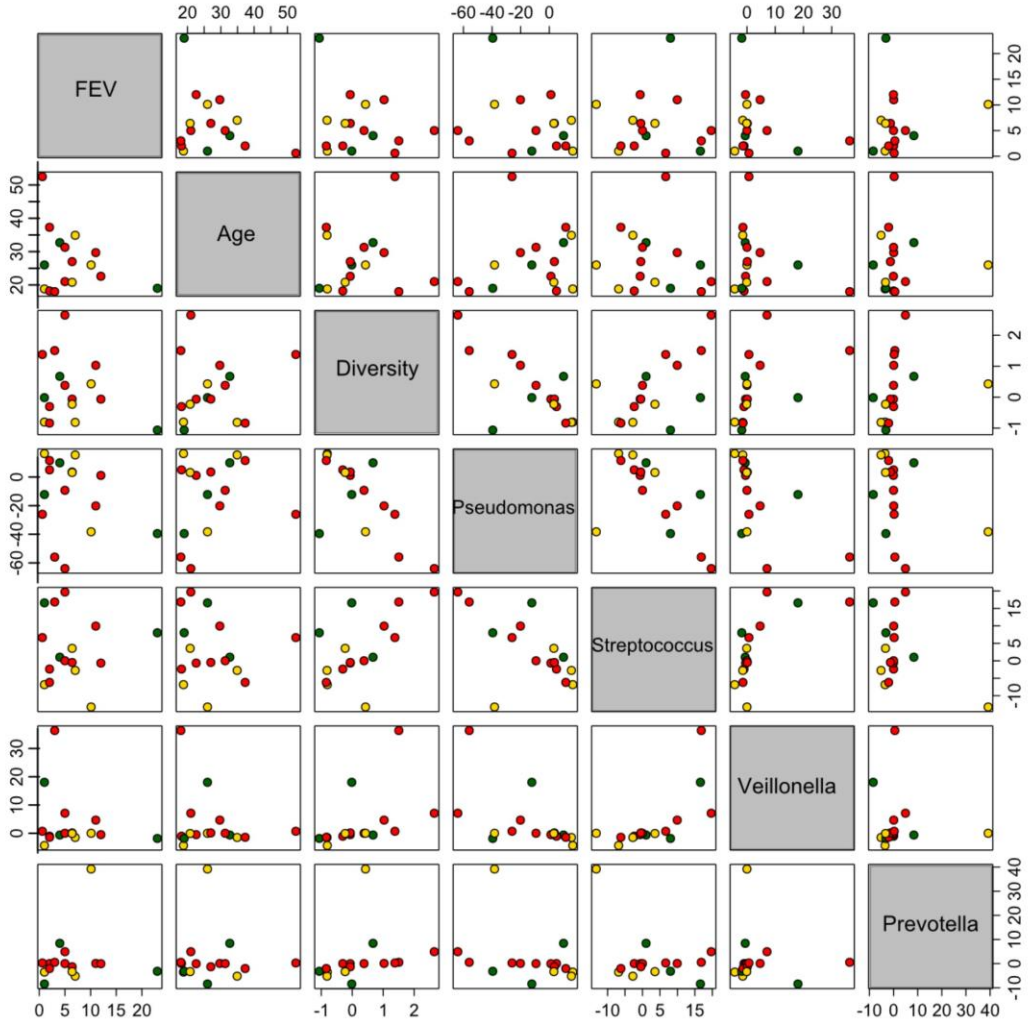
The diagonal of the matrix lists the variables (grey boxes) compared in the scatterplots. Variables considered were FEV_1, age, Shannon diversity and relative abundance of the most abundant bacterial genera. Each panel in the matrix contains a scatterplot for a pair of variables. Each dot in the scatterplots corresponds to the measurements of the variable for an individual subject and is coloured according to the hierarchical cluster (H, M, L) assigned to the subject at TP-1 . The scatterplots in the upper triangle (above the diagonal) or lower triangle (below the diagonal) illustrate the relationships of the variables at TP-1 and TP-3, respectively. The relationship between the relative abundance of *Pseudomonas* and microbial diversity was negatively correlated (Pearson, r=-0.87, p<0.01). A positive correlation was seen between microbial diversity and *Streptococccocus* (r=0.63, p<0.01) and *Prevotella* (r=0.6, p=0.01).

Figure 3-3 Changes in CF sputum microbiota between TP-1, TP-2 and TP-3.



A. Shannon index, demonstrating changes in overall microbial diversity B. Changes in the relative abundance of *Pseudomonas* C. Circle plots demonstrating changes in global community structure at TP-1 (left), TP-2 (middle), TP-3 (right). Each ring represents a taxonomic rank (phylum (inner-most), class, order, family, genus (outer-most)). The colour code for the rank phylum is illustrated in the legend.

Figure 3-4 Scatterplot matrix visualizing pairwise correlations of variables participant demographics and change in microbial composition between TP-1 and TP3



The diagonal of the matrix lists the variables (grey box) that were compared in the scatterplots. Variables included were; improvement in FEV_1, age, change in Shannon diversity and change in relative abundance of selected bacterial genera. The change was determined by calculating the difference of the Shannon diversity or relative abundance of the genus between TP-1 and TP-3. Each dot in the scatterplots corresponds to the measurements of the variable for an individual subject and is coloured according to the hierarchical clustered (H, M, L) assigned to the subject at TP-1. The change in microbial diversity demonstrated a negative correlation with the relative abundance of Pseudomonas (Pearson, r=-0.76, p<0.001) and a positive correlation with the relative abundance of Streptococcus (r=0.6, p=0.01).

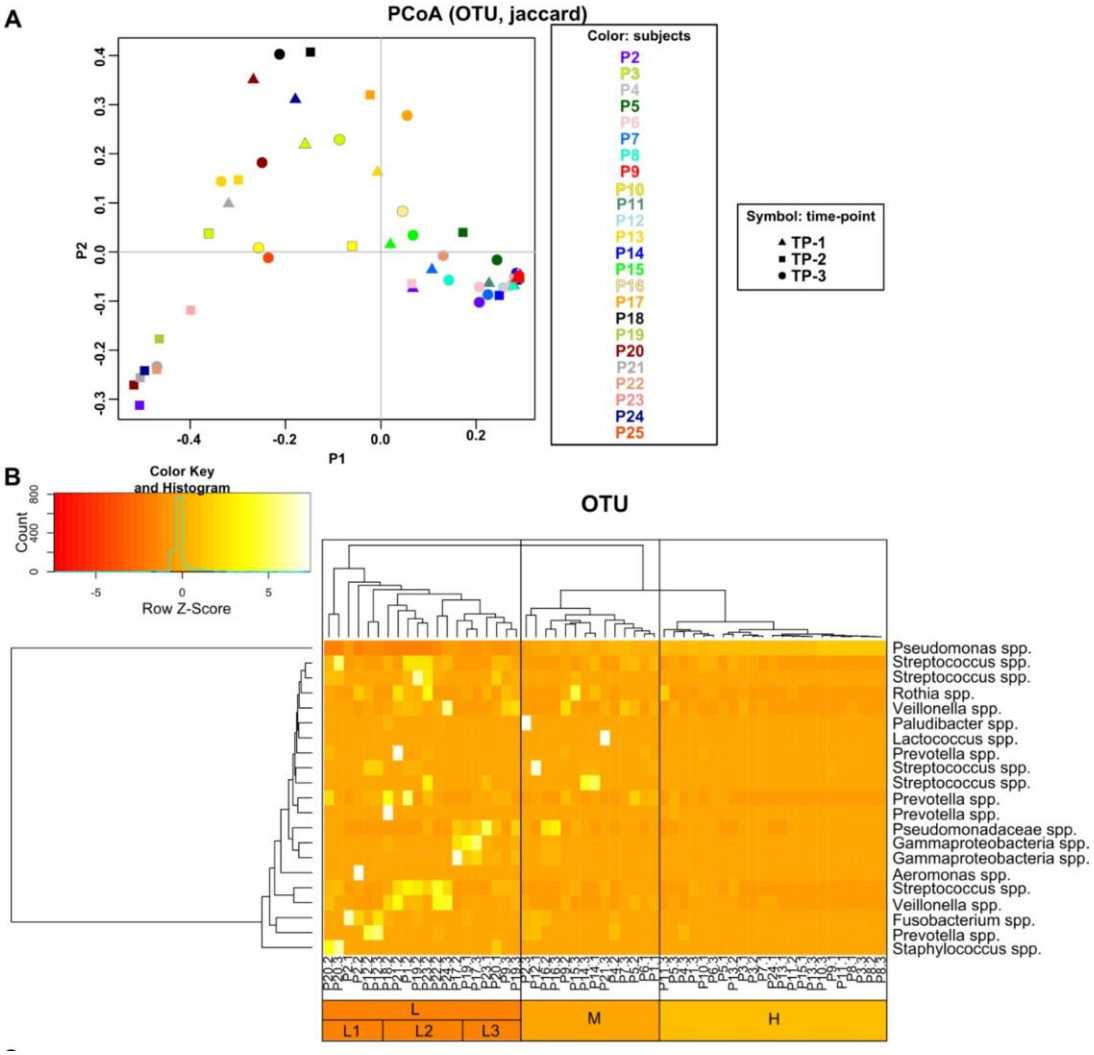
At TP-3, overall microbial diversity (Shannon index) and *Pseudomonas* relative abundance were similar to TP-1, suggesting a return to pre-treatment community composition (Figure 3.3). The change in overall microbial diversity correlated negatively with the change in abundance of *Pseudomonas* ($r=-0.7$, $p<0.01$) and positively with change in abundance of *Streptococcus* ($r= 0.6$, $p<0.05$) (Figure 3.4).

A principal coordinates analysis demonstrated samples from TP-1 and TP-3 clustered together, however, TP-2 formed a distant, separate cluster (Figure 3.5). This clustering was significant; comparison of intra- and inter-group Jarrard distances of TP-1 versus TP-3 were not significant ($p=0.75$), but significant between TP-1 versus TP-2 and TP-2 versis TP-3 ($p<0.01$).

We did not observe any significant associations between the changes in abundance of individual genera and improvement in FEV_1 between TP-1 and TP-3. A negative relationship between relative abundance of *Pseudomonas* and FEV_1 was observed at TP-3, but did not reach statistical significance ($r=-0.46$, $p=0.07$).

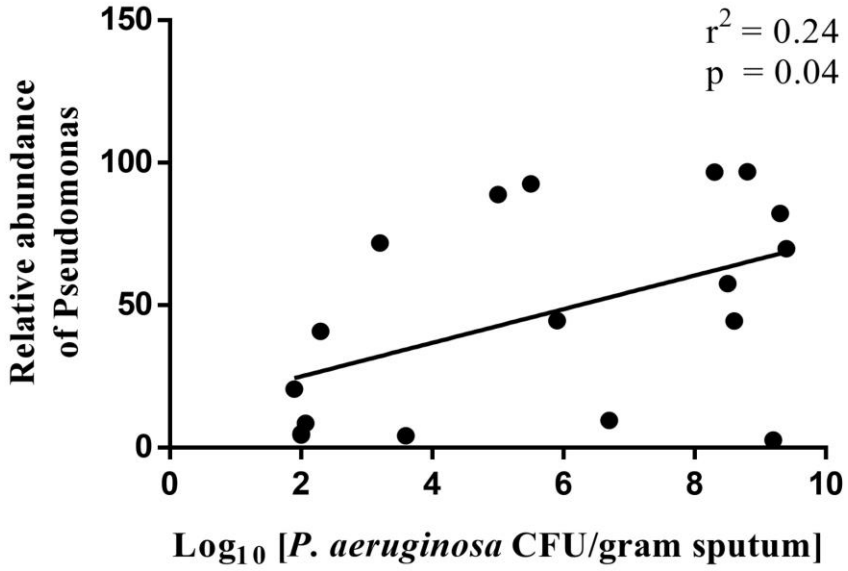
In the nine subjects for whom *P. aeruginosa* quantification was performed, there was a weak, positive correlation between *P. aeruginosa* load and the relative abundance of Pseudomonas at the genus level ($r^2 = 0.24$, $p= 0.04$) (Figure 3.6).

Figure 3-5 PCoA (A) and hierarchical clustering of CF sputum samples based on OTU profiles (B).



A. PCoA of the sputum microbiome showing a shift in the microbiota at TP-2 (rectangle). TP-1 (triangle) and TP-3 (circle) for some subjects clustered together indicating a return to the initial community composition. B. Only OTUs that had a relative abundance >10% in at least one sample are shown. Hierarchical clustering demonstrated grouping of samples based on the abundance of OTUs related to *Pseudomonas spp*: (L) Mixed community consisting of low levels of *Pseudomonas* and presence of several other bacterial groups, (M) Intermediate abundance of *Pseudomonas* and only minor other bacterial groups, (H) *Pseudomonas*-dominated samples. Group L can further be subdivided according to the prevalence of *Staphylococcus* and *Fusobacterium* (L1), *Streptococcus* (L2) and other unknown *Gammaproteobacteria* species (L3).

Figure 3-6 Pearson's correlation demonstrating the relationship between the relative abundance of Pseudomonas (genus) and *P. aeruginosa* colony forming units (CFU) per gram of sputum, determined by RT-qPCR.

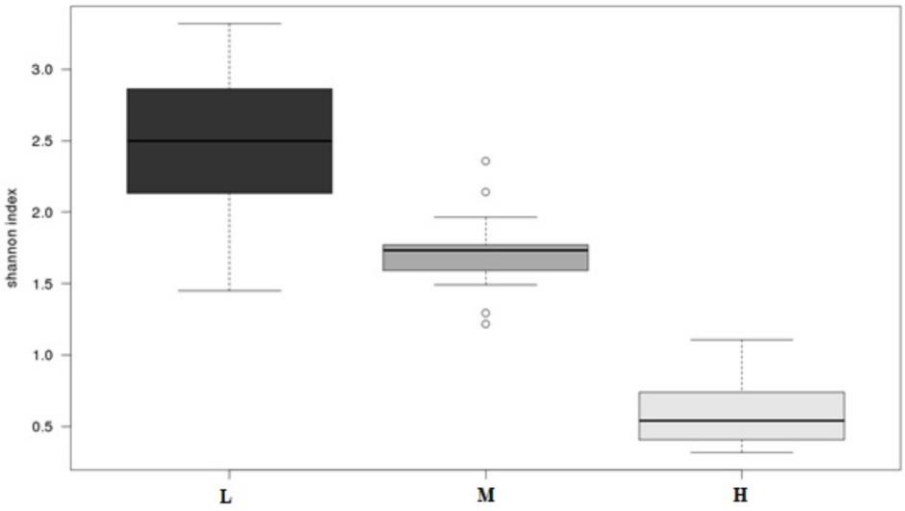


3.4.4 Sub-group analysis based on the relative abundance of *P. aeruginosa.*

Clustering of OTUs based on the relative abundance of *Pseudomonas spp* revealed three distinct groups: (L) low abundance of *Pseudomonas spp*. (<40%) with a complex community structure and presence of various other bacterial genera, (M) medium abundance of *Pseudomonas spp*. (40 to <75%), and (H) high abundance of *Pseudomonas spp.* (≥75%) (Figure 3.7).

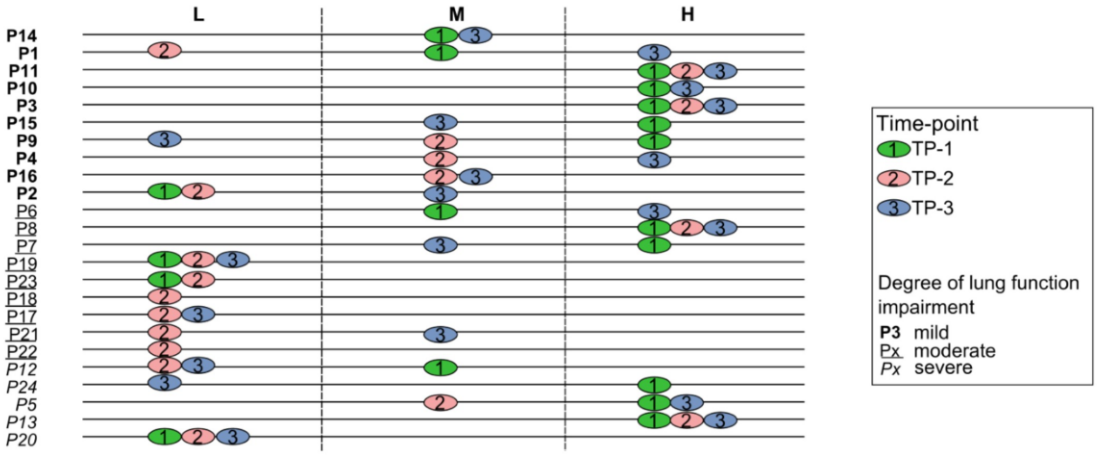
Samples from subjects with severe disease tended to cluster in the H and M categories, whereas samples from subjects with mild to moderate disease clustered in the L category (Figure 3.8). Antibiotic treatment rarely resulted in a change in a subject's category at the end of treatment, and changes in the relative abundance of *Pseudomonas* in response to treatment were not associated with an improvement in lung function.

Figure 3-7 Community diversity (Shannon index) of samples by hierarchical clustering group.



The greatest diversity is seen in the L group, with low diversity seen in the H group.

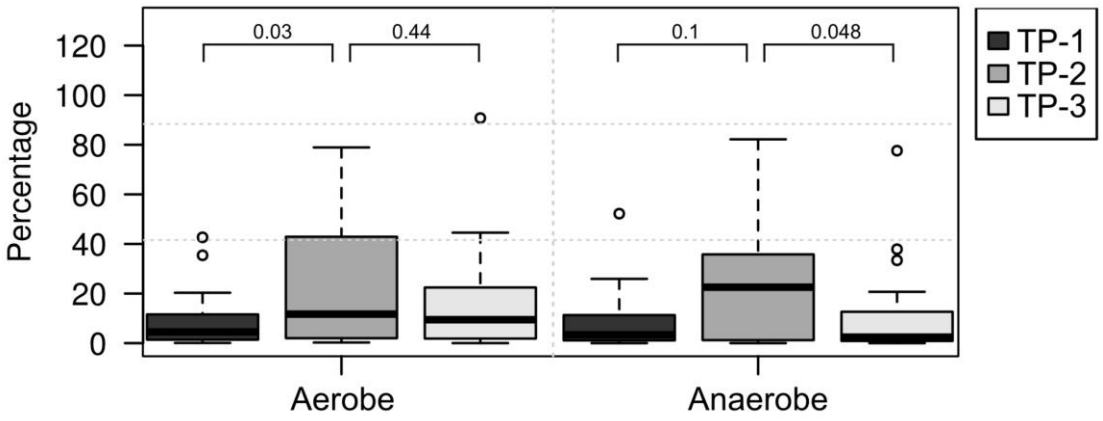
Figure 3-8 Categorization of subjects according to identified sputum microbiota groups.



The time-point (TP-1: green, TP-2: red, TP-3: blue) of the samples for each subject is categorized according to the groupings retrieved from hierarchical clustering. Subjects are stratified by order of the degree of lung function impairment (FEV1 percentage predicted), with most severe impairment at the bottom and least severe at the top (**Px**: mild, Px: moderate, *Px***:** severe lung function impairment). Samples from subjects with a severe CF lung function impairment tended to cluster in group H or M, whereas samples from subjects with mild-moderate impairment clustered in the L group. The microbial diversity (Shannon index) of samples by hierarchical cluster is presented in Figure 3.7.

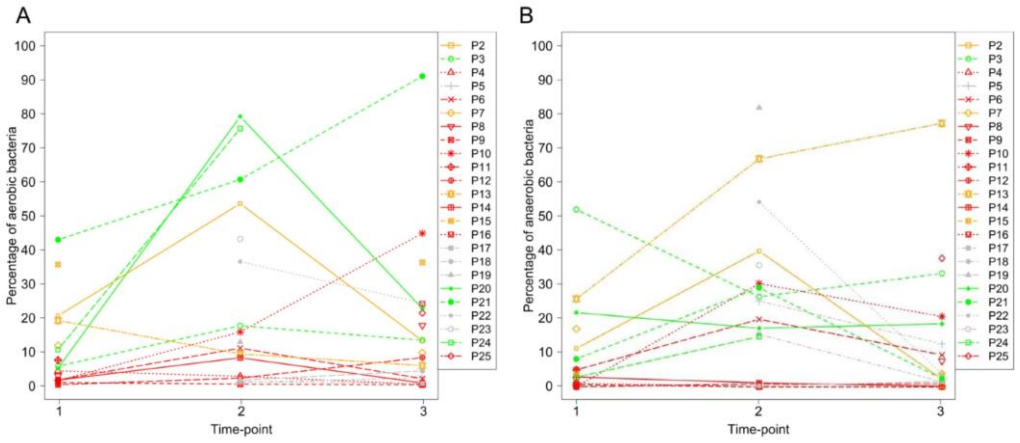
Non-*Pseudomonas* genera were categorised as obligate anaerobes or aerobes/facultative anaerobes (Figure 3.9). By TP-2 the relative abundance of aerobic bacteria increased (median, TP-1: 4.5% of 16S sequences; TP-2: 11.7%; p=0.03; FDR=0,049) and a similar trend was observed in the abundance of anaerobic bacteria (median, TP-1: 3.4%, TP-2: 22.6%, p=0.1). A reversal in these trends was seen between TP-2 and TP-3 (aerobes TP-3, median 9.5%, paired t-test between TP-2 and TP-3 p=0.44; anaerobes TP-3, median: 2.4%, p=0.048, FDR=0.1) and at TP-3, the sputum community composition once again resembled TP-1 (Figure 3.9).

Figure 3-9 Relative abundance (median) of aerobic and anaerobic genera in CF sputum samples after exclusion of reads assigned to *Pseudomonas*.



There was a significant increase in the relative abundance of aerobic bacteria between TP-1 and TP-2, with a similar trend seen in anaerobic bacteria, followed by a significant decrease in anaerobic bacteria between TP-2 and TP-3. P-values were calculated using paired t-test. Changes in the relative abundance of aerobic and anaerobic genera in response to treatment in individual subjects is presented in Figure 3.10.

Figure 3-10 Relative abundance of, A. aerobic and, B. anaerobic bacteria for each CF subject.



The colour indicates the hierarchical clustering group of the subjects at time-point 1 (red: group H, orange: group M, green: group H, grey: unknown [no sample at time-point 1])

3.5 Discussion

In this study, we demonstrate a significant perturbation of the airway microbiome over the first 72 hours of IV antibiotic treatment for pulmonary exacerbations in adult subjects with CF. This perturbation was characterised by a reduction in the dominance of *Pseudomonas* and an accompanying increase in microbial diversity. These changes were, however, short-lived, with bacterial community composition resembling that of the initial profile after one week of antibiotic treatment. These novel findings suggest that disturbance of the bacterial composition of the CF airway in response to IV antibiotics is transient, and potentially challenge current antibiotic management strategies for CF pulmonary exacerbations.

Evidence in the literature to support the application of culture-independent techniques to examine the effect of antibiotic therapy on the CF airway microbiome remains limited. Tunney *et al.* have demonstrated that despite total bacterial numbers being reduced in response to antibiotics (culture-based assessment), there was relative stability in overall community composition as assessed by terminal restriction fragment length polymorphisms analysis when sputum samples were collected from subjects with CF before and after

treatment of a pulmonary exacerbation(136). More recently, Daniels *et al* examined the impact of antibiotics on CF sputum microbial diversity in a group of adult CF subjects(137). Sputum samples collected following the initial 72 hours of antibiotic therapy were compared with samples collected during a period of clinical stability prior to the exacerbation and after 10-14 days of treatment. In comparison to samples collected at 72 hours, samples collected at 10-14 days demonstrated an increase in relative abundance of *Pseudomonas* species compared with non-pseudomonads accompanied by a reduction in community diversity(137). The authors concluded that antibiotic therapy was exerting a significantly greater effect on bacterial species other than *Pseudomonas*, culminating in the dominance of *P. aeruginosa*.

By examining microbial diversity at the onset of the exacerbation, prior to the administration of antibiotics, we have advanced the findings of Daniels *et al* and shown that bacterial communities appear most susceptible to IV antibiotics at the beginning of antibiotic therapy. In addition to performing sampling prior to the onset of antibiotics, a number of important differences between the current study and that of Daniels and colleagues should be highlighted. Firstly, in the earlier study, subjects were treated with a range of oral, inhaled and IV antibiotics which may have affected the ability to pick up consistent changes in community composition, whereas the subjects reported here were consistently treated with conventional IV antibiotic combinations. Additionally, sample processing and sequencing techniques differed between the two studies, most notably the samples in Daniel's study were treated with propidium monoazide (PMA) to cross-link DNA from non-viable bacteria, a technique we did not employ. Nevertheless, both studies confirm that community composition remains essentially unchanged after at least a week of IV antibiotic therapy, which, given the early alterations in the microbiome that we observed, suggests that current treatment practices may need to be revised(266, 267).

The reduction in relative abundance of *Pseudomonas* from day 1 to days 3-4 suggests initial preferential killing of *Pseudomonas* in response to IV antibiotics. Recrudescence of *P. aeruginosa* infection under antibiotic pressure after this early response may occur as a result of increased replication of an inherently antibiotic resistant sub-population, or through population-wide adaptive mechanisms involving the up-regulation of antibiotic resistance genes, which may occur rapidly in the treatment course(268, 269)**.** Whilst alternative explanations for the clinical improvements (reduction in respiratory symptoms and subjective wellbeing) in subjects beyond the first few days of treatment should also considered,

including the impact of adjunctive therapies, such as inhaled mucolytics, airway clearance techniques, rehydration and nutritional support(127, 134, 245), our findings challenge the convention of prescribing the same IV antibiotic combination for more than a few days at a time. A potential new strategy may involve the rapid cycling of different antibiotic regimes during treatment, but this would require investigation in large-scale, adequately powered randomised and blinded clinical trials.

To explore the effect of antibiotics on bacterial species other than *P. aeruginosa*, we examined microbial community changes with *Pseudomonas* reads excluded. Contrary to the earlier study by Daniels *et al*, the relative abundance of both aerobes and anaerobes increased in the early stages of treatment, suggesting antibiotic therapy was having a lesser impact on the other bacterial species present compared to *Pseudomonas*. The effects of IV antibiotics in adults not infected with *P. aeruginosa* who are experiencing an acute exacerbation warrants further investigation, particularly as these individuals represent an increasing group of subjects transitioning from paediatric to adult care(270).

Greater sputum microbial diversity in our study subjects was positively associated with the abundance of *Streptococcus*, accompanied by a reduction in the relative abundance of *Pseudomonas*. These findings are consistent with a previous cross-sectional study in which microbial profiles of stable CF outpatients and inpatients were compared(271). In this earlier report, three distinct sub-groups of CF subjects were identified by the relative abundance of *Streptococcus* and *Pseudomonas* in sputum. Importantly, outpatients with high *Streptococcus* and low *Pseudomonas* abundance had greater lung function stability over time. In our study, sputum samples with a high relative abundance of *Pseudomonas* and low community diversity formed a cluster, predominantly in subjects with severe lung disease. However, in contrast to other studies, we were unable to confirm a reduction in microbial diversity with increasing age and severity of lung disease in the CF subjects we studied(84, 266).

One potential limitation of our study is that PMA was not used to exclude DNA from non-viable bacterial cells in the sputum samples prior to analysis(272). The use of PMA has been advocated due to appropriate theoretical concerns that molecular based techniques may identify both viable and non-viable bacteria and limit the ability to detect changes in bacterial number in antibiotic treatment responses studies(273). However, the reduction in the relative abundance of *Pseudomonas* at day 3 that we observed would not be consistent with the

inclusion of non-viable organisms(272). The use of PMA remains a topic of debate and the method carries its own potential drawbacks, including a limited ability to expediently penetrate purulent, non-homogenised sputum prior to the death of resident organisms deep within the sputum sample. Furthermore, the extended processing time involved may result in less hardy bacteria (e.g. anaerobes) preferentially dying *ex vivo,* while more robust bacteria proliferate and skew the true bacterial composition, even if samples are maintained at 4°C(74, 136).

Our conclusions are based on changes in relative abundance and not quantitative bacterial load. We have previously performed enumeration of *P. aeruginosa* number by RT-qPCR and demonstrated heterogeneous changes in bacterial numbers in response to antibiotics(265). The findings for some subjects in this earlier work were consistent with the current study, with an early reduction in *P. aeruginosa* numbers, being followed by a recrudescence by the end of the first week of treatment(265). Quantification of *P. aeruginosa* by RT-qPCR in nine subjects from the current study revealed a positive correlation between *P. aeruginosa* load and the relative abundance of *Pseudomonas* at the genus level (Figure 3.6) and supports conjecture that changes in community composition in the CF lung reflect changes in *P. aeruginosa* concentration, however, this relationship was weak. Matching changes in community structure with enumeration of individual bacterial species is complex and changes in individual species, may not be equivalent to changes at the level of the genus. Future advances in pyrosequencing technology may allow for identification and enumeration of bacteria at the species level, which will substantially advance understanding of the dynamics of the lung microbiome.

Approximately 3% of all obtained sequences in our study could not be assigned to a genus, which is consistent with the findings of others(274). The success of read assignment is dependent on read length, primer pairs and the particular reference database applied to the analysis(219). To date, there is no universal method of analysis agreed upon, which further confounds direct comparisons of the human microbiome between studies.

3.6 Conclusions.

We have demonstrated for the first time that the relative abundance of *P. aeruginosa* falls rapidly in subjects with CF receiving IV antibiotics for pulmonary exacerbations, and that this is accompanied by an increase in microbial diversity. This effect was not maintained beyond the first week of treatment. These findings have implications for how IV antibiotic treatment should be employed for exacerbations and for how long. Future clinical trials should consider the impact of IV antibiotics on the whole lung microbiome in CF, how changes in microbial community composition relate to reductions in absolute bacterial counts and in turn, how these parameters relate to the clinical response to treatment.

Chapter 4. Lymphocyte Phenotype in CF

4.1 Abstract.

Background: Primary defects in host immune responses have been hypothesised to contribute towards an inability of subjects with cystic fibrosis (CF) to effectively clear pulmonary infections. Innate T-lymphocytes provide rapid pathogen-specific responses prior to the development of classical MHC class I and II restricted T-cell responses and are essential to the initial control of pulmonary infection. We aimed to examine the relationship between peripheral blood lymphocyte phenotype and clinical outcomes in adults with CF.

Methods: We studied 41 subjects with CF and 22, age matched, non-smoking healthy control subjects. Lymphocytes were extracted from peripheral blood samples and phenotyped by flow-cytometry. Lymphocyte phenotype was correlated with sputum microbiology and clinical parameters.

Results: In comparison to healthy control subjects, mucosal associated invariant T (MAIT)-lymphocytes were significantly reduced in the peripheral blood of subjects with CF (1.1% versus 2.0% of T-lymphocytes, P=0.002). MAIT cell concentration was lowest in CF subjects infected with *P. aeruginosa* and in subjects receiving treatment for a pulmonary exacerbation. Furthermore a reduced MAIT cell concentration correlated with severity of lung disease.

Conclusion: Reduced numbers of MAIT cells in subjects with CF were associated with *P. aeruginosa* pulmonary infection, pulmonary exacerbations and more severe lung disease. These findings provide the impetus for future studies examining the utility of MAIT cells in immunotherapies and vaccine development. Longitudinal studies of MAIT cells as biomarkers of CF pulmonary infection are awaited.

4.2 Introduction.

Cystic fibrosis (CF) pulmonary disease is typified by a vicious cycle of bacterial infection and exuberant, but ineffective host immune response(275). The inability of the intense inflammatory response to clear infection has led to speculation that intrinsic immune defects may contribute to the persistence of pathogens in CF(25). At the level of the airway lumen, the cellular immune response is dominated by activated neutrophils. However, in contrast, airway epithelial biopsies demonstrate a profound T lymphocyte (T-cell) infiltrate, supporting an important role for adaptive immune responses in the orchestration of a sustained inflammatory response(47).

To date, studies of peripheral, adaptive immune responses in CF have largely focused on the classic dichotomy of T-helper (Th)-1 and Th-2 responses(49). These early studies suggested a skew towards a Th2 in most CF subjects with *P. aeruginosa* infection, which resulted in increased pulmonary inflammation and disease progression(49-51). The activation of the "classical" adaptive immune response involves antigen recognition, followed by T-cell recruitment and clonal expansion at the site of infection. Consequently, there is a time lag between the host's recognition of the presence of a pathogen and the development of an effective, adaptive immune response. In recent times, an increasing number of unconventional "innate" T-cell populations have been described (including γ/δ, semi-invariant natural killer (iNKT) and M3-restricted T-cells), which are capable of mounting a more immediate response to pathogens than was previously thought possible. Mucosal associated invariant T (MAIT) cells are a recently described sub-class of innate T-cells, which can be differentiated from other T-cells by the presence of an evolutionary conserved T-cell receptor (TCR) (Vα7.2-Jα33). MAIT cells recognise bacterial and fungal metabolites presented on the major histocompatibility complex (MHC) related protein-1 (MR1) (including the common CF pathogens *Pseudomonas aeruginosa* and *Staphylococcus aureus*), but not viruses(69, 70). These "innate" T-cell populations provide rapid pathogen-specific responses prior to the development of classical MHC class I and II restricted T-cell responses and importantly may also provide a sustained cytokine response in chronic infection(71, 72).

To date, there is limited knowledge of how changes in circulating lymphocyte populations may relate to pulmonary infection in CF(276). In this study we performed extensive phenotyping of peripheral blood mononuclear cell populations (PBMCs) obtained from

subjects with CF and compared these profiles to those in healthy, age matched, controls. Our particular focus was on the correlation of γ/δ and MAIT innate T-cell values with clinical and microbiological parameters.

4.3 Methods.

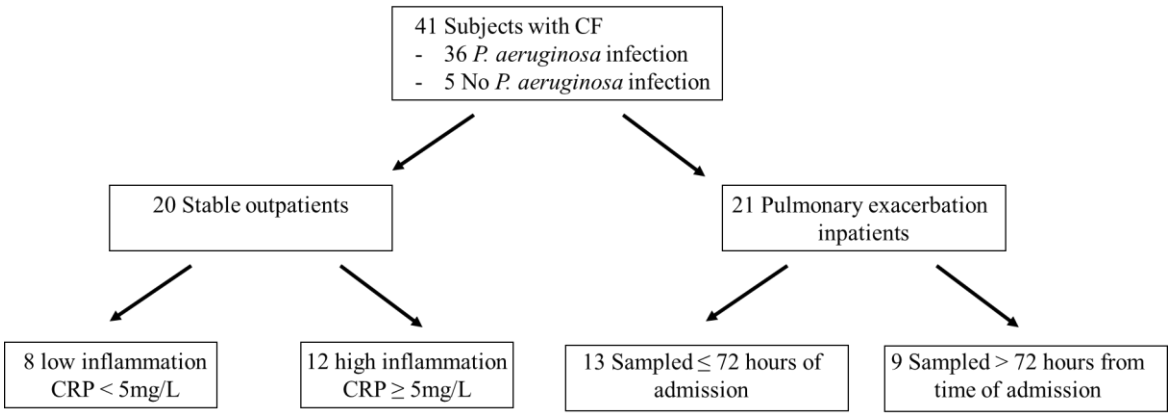
4.3.1 Participants and sample collection.

Forty-one subjects with CF attending the Adult CF Centre, The Prince Charles Hospital, Queensland, Australia and 22 age-matched, non-smoking, healthy control subjects each supplied a single venous blood sample.

In subjects with CF, total white cell count (WCC), C-reactive protein (CRP) and clinical demographics including, age, CF genotype, lung function, body mass index, pulmonary exacerbation frequency and pulmonary pathogens (based on standard sputum microbiological testing) were recorded. Longitudinal rate of decline in forced expiratory volume in one second (FEV_1) was determined in CF subjects by means of linear regression analysis (limited to subjects with at least five FEV_1 measurements recorded over a minimum surveillance period of 2 years).

To explore the effect of pulmonary exacerbations on lymphocyte concentrations a sub-set of 13 “stable” CF subjects (stable respiratory symptoms and a CRP < 5 mg L^{-1} at time of blood collection) were compared to eight CF subjects in whom blood was collected within 72 hours of admission to hospital for the intravenous antibiotic treatment of a “pulmonary exacerbation”, defined as increased respiratory symptoms (cough, sputum volume or purulence, dyspnoea) (Figure 4.1).

Figure 4-1 Flow diagram of subjects included in sub-group analysis



Ethics approval was obtained from The Prince Charles Hospital, Queensland, Australia, Human Research and Ethics Committee (HREC/11/QPCH/36 and HREC2008:2885) and all subjects provided written, informed consent.

4.3.2 Separation and storage of peripheral blood mononuclear cells (PBMCs).

Twelve millilitres of venous blood was collected into lithium heparinised tubes and separated into plasma and cellular components. The cellular component was re-suspended in RPMI (Gibco®) + 2% heat inactivated Foetal Calf Serum (HiFCS) and PBMCs separated by means of Histopaque® 1.077 (Sigma-Aldrich®) density gradient separation, as per the manufacturer's protocol. Following separation, PBMCs were washed twice, re-suspended in RPMI and 15% dimethyl sulfoxide (DMSO), gradually frozen to -80°C and transferred to storage in liquid nitrogen for later batch analysis (concentration 5-20x10^6 cells/ml).

4.3.3 Flow Cytometry.

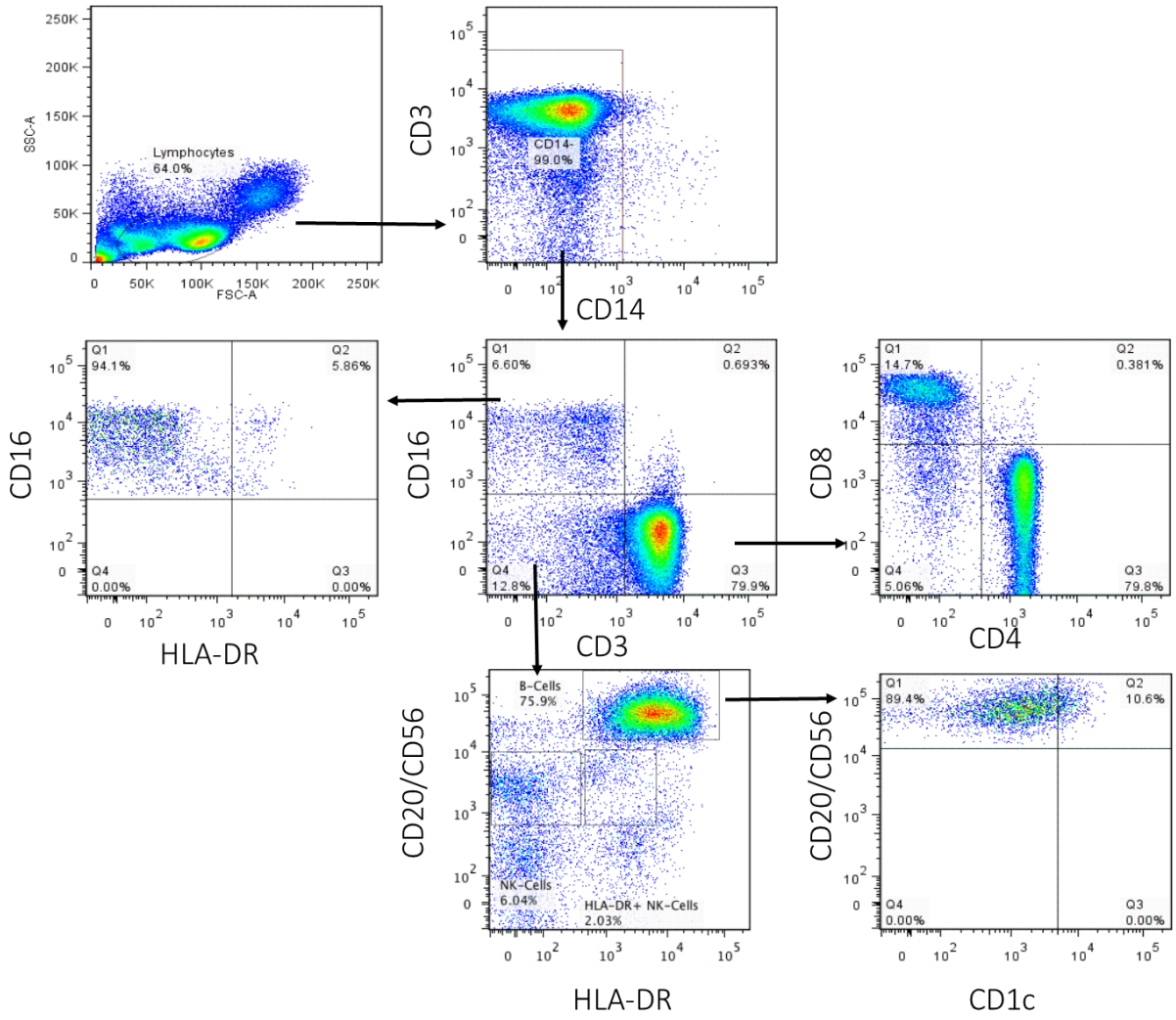
PBMCs were rewarmed and re-suspended by drop-wise addition of 10ml of RPMI+2%HiFCS, washed twice and re-suspended in phosphate buffered saline (PBS) with 1% HiFCS (FACS buffer). A cell count was performed and the volume adjusted to obtain a cell concentration of 10 x10^6 cells/ml.

One hundred micro-litre aliquots of cells were incubated with each of two antibody staining panels as follows:

Panel 1 [adapted from (277)]: FITC anti-human CD16, Pacific Blue anti-human CD14, APC anti-human CD1c, Alexa Fluor700 anti-human CD3, APC/Cy7 anti-human HLA-DR, PE/Cy7 anti-human CD56, PE/Cy7 anti-human CD20, Anti-CD8 antibody – PE Texas (Abcam) and V500 anti-CD4 (BD biosciences) were added to 100µL of cells and incubated in the dark for 15 minutes. Cells were washed twice in 1ml of FACS buffer and fixation was performed by incubation with 500µl of Cytofix (BD biosciences) for 10 minutes. Finally, samples were washed and suspended in 300µl of FACS buffer (example staining panel Figure 4.2).

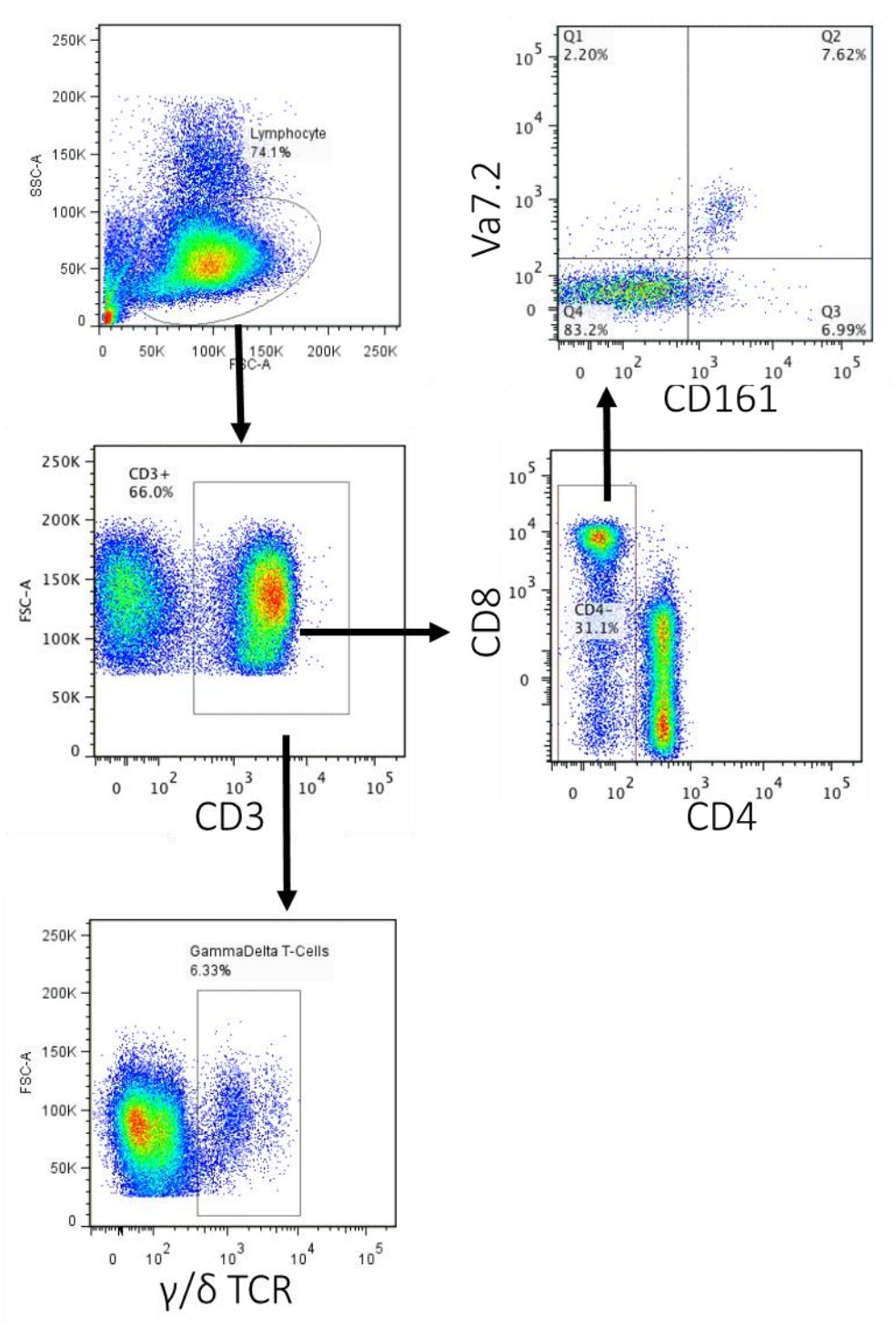
Panel 2: Surface staining was performed by incubation with V500 anti-CD4, FITC anti-TCR Va7.2, PerCP/Cy5.5 anti-CD161, APC/Cy7 anti-CD3, PE anti-TCR γ/δ, PE Texas red Anti-CD8 for 15 minutes at room temperature in the dark. Cells were washed twice in 1ml of FACS buffer, fixed and resuspended in 300µl of buffer solution (example staining panel Figure 4.3).

Figure 4-2 Representative flow-cytometry gating plots for determination of Major Lymphocyte sub-sets.



Cell Characteristic; Lymphocytes (Appropriate forward and side scatter properties and CD14-), T-Cells (CD3+/CD16-), CD3+/CD16+ cells, NK-Cells (CD16+/CD3-/HLA-DR-, CD16-/CD3-/CD56+/HLA-DR- and CD16-/CD3-/CD56+/HLA-DR+), CD4+ T-Cells (CD3+/CD16-/CD4+/CD8-), CD8+ T-Cells (CD3+/CD16-/CD4-/CD8+), CD4+/CD8+ T-Cells (CD3+/CD16-/CD4+/CD8+), CD4-/CD8- T-Cells (CD3+/CD16-/CD4-/CD8+), B-Cells (CD3-/CD16-/CD20+/HLA-DR+)

Figure 4-3 Representative flow-cytometry gating plots for determination of MAIT cells and γ/δ T-Cells.



Cell Characteristics: MAIT Cells (CD3+/CD4-/TCR Va7.2+/CD161+), γ/δ T-Cells (CD3+/TCR γ/δ+).

Unless stated otherwise, antibodies were obtained for Biolegend®, San Diego. Antibody titration was performed to optimise antibody-cell concentration prior to testing.

Sample analysis was performed on a Fortessa IV flow cytometer (BD Biosciences). A lymphocyte gate was set based on forward and side scatter properties and a minimum of 50,000 gated events were capture for each sample.

MAIT cells were defined as $CD3^+/CD4^-/CD8^+$or-/$CD161^+$/TCR $Va7.2^+$(278).

For subjects with CF, automated haemocytometer, absolute lymphocyte counts were obtained and T-cell sub-types were considered as both, absolute numbers of cells per mL of blood and percentage of the whole T-cell population.

Data analysis was performed using Flowjo version 7.6 (Treestar).

4.3.4 Statistical Analysis.

Statistical analysis was performed using PASW, version 18 (SPSS Inc. Chicago IL, USA) and Graph-pad Prism, version 6. Between group differences in PBMC populations were examined using student's t-test or Mann-Whitney U test. Shapiro-Wilk test and q-q plots were used to determine normal distribution of continuous variables. Non-normally distributed continuous variables (WCC, CRP, MAIT cell absolute count and percentage, B-cell count, NKT-cell and NK-cell percentage) were natural logarithm transformed and Pearson's correlation used to determine relationships between variables. A p-value <0.05 was considered to represent statistical significance.

4.4 Results.

The characteristics of subjects with CF and healthy controls are provided in Table 4.1. Thirty-six of the 41 subjects with CF had chronic pulmonary infection with *P. aeruginosa* (either in isolation or in combination with another CF respiratory pathogen) on routine microbiological cultures. The remaining five subjects did not have *P. aeruginosa* infection on current, or previous sputum cultures (See Appendix 3.1 for complete sputum microbiological data).

Table 4-1 Subject demographics

	Healthy Controls	Cystic Fibrosis	P value
Sex (Female:Male)	11:11	17:24	0.5
Age (years)	26 (25 - 32)	28 (22 - 32)	0.9
BMI ($kg/metre^2$)	24.6 (21.4 – 28.3)	23.5 (20.5 – 25.8)	0.3
FEV_1 (Litres)	4.1 (3.5 -4.7)	2.2 (1.4 – 2.5)	<0.001
FEV_1 % Predicted (%)	110 (101 – 120)	58 (37 - 75)	<0.001
FVC (Litres)	4.9 (4.4 – 5.7)	3.1 (2.6 – 4.1)	<0.001
FVC % Predicted (%)	107 (95 – 112)	76 (58 - 84)	<0.001
CFTR Genotype - F508del Homozygotes - F508del Heterozygotes - Other mutations		 20 18 3	
Sputum Microbiology* - *Pseudomonas aeruginosa* - *Staphylococcus aureus* - *Aspergillus fumigatus* - *Haemophilus influenzae* - *Stenotrophomonas maltophilia* - *Chryseobacterium indologenes* - *Scedosporium apiospermum* - *Mycobacterium intracellulare* - *Burkholderia gladioli* - *Achromobacter xylosoxidans* - Methicillin resistant *S. aureus*		 36 12 5 4 3 2 2 1 1 1 1	

Data presented as median (interquartile range). *Summary data, subjects may have had more than one pathogen isolated in sputum, individual microbiological data available in Appendix 3.1.

A greater number of subjects in the pulmonary exacerbation group were male, these subjects were also older, with more severe lung disease, when compared to the stable subjects (Table 4.4).

Comparison of lymphocyte sub-sets between groups, demonstrated a reduction in the percentage of MAIT cells in subjects with CF, compared to healthy controls (median 1.1% versus 2.0%, p=0.002), with an accompanying increase in the percentage of γ/δ T-cells (median 10.4% versus 6.4%, p=0.012). CF subjects also displayed reduced percentages of NK-cells (median 9.5% versus 13.1%, p=0.013). The percentage of cells in all of the other major lymphocyte sub-sets was similar between groups (Table 4.2).

4.4.1 Relationship between MAIT cells, microbiological and clinical parameters in subjects with CF.

Absolute MAIT cell concentrations and the proportion of T-cells that were MAIT cells (MAIT cell percentage), in the five subjects without *P. aeruginosa* infection were significantly higher than in patients with chronic *P. aeruginosa* infection (Table 4.3 and Figure 4.4A). MAIT cell percentages in subjects not infected with *P. aeruginosa* were similar to healthy controls subjects. No difference was seen in the MAIT cell percentage of subjects with a *P. aeruginosa* infection, based on their co-pathogen (Figure 4.5).

Absolute blood MAIT cell counts in the sub-group of stable CF subjects were higher, when compared to subjects sampled early in the course of treatment for a pulmonary exacerbation (Figure 4.4B). However, the MAIT cell percentage was similar between stable and pulmonary exacerbation subjects (Table 4.4).

Table 4-2 Comparison of lymphocyte sub-sets between CF and healthy control subjects.

Lymphocyte Population (size gated, CD14-)	CF (n=41)		Non-CF (n=22)		P-Value
T-Cells (CD3+CD16-)	**72.6**	**(68.1 – 79.6)**	**74.9**	**(67.9 – 79.0)**	0.6
- CD4+CD8-	- 65.2	(56.1 – 70.5)	- 65.1	(59.9 – 71.4)	0.6
- CD8+CD4-	- 25.7	(21.1 – 32.5)	- 27.4	(22.7 – 32.8)	0.6 **0.002**
- MAIT Cells (CD161+, TCR Va7.2+)	- 1.1	(0.4 – 1.9)	- 2.0	(1.4 – 3.1)	**0.012**
- γ/δ T-cells (TCR γ/δ+)	- 10.4	(6.5-13.4)	- 6.4	(4.6 – 9.4)	0.1
- CD4+CD8+	- 0.3	(0.2 – 1.0)	- 0.4	(0.3 – 1.7)	0.3
- CD4-CD8-*	- 2.1	(0.9 – 3.3)	- 1.6	(1.1 – 2.3)	
B-Cells (CD3-CD16-CD20+HLA-DR+)	**9.5**	**(4.9 – 14.3)**	**4.8**	**(3.9 – 8.7)**	0.1
- Non-Resting (CD1c-)	- 64.6	(57.0 – 74.4)	- 71.6	(60.5 – 75.1)	0.5
- Resting (CD1c+)	- 35.0	(24.8 – 43.0)	- 28.1	(25.0 – 38.3)	0.4
NK-Cells (CD3-)	**9.5**	**(6.9 – 12.2)**	**13.1**	**(8.0 – 18.0)**	**0.013**
- CD16+ CD56dim HLA-DR-	- 75.1	(61.0 – 86.7)	- 88.0	(84.7 – 92.6)	**0.001**
- CD16- CD56+ HLA-DR-	- 8.9	(6.1 – 13.2)	- 6.1	(4.5 – 9.1)	**0.016**
- CD16- CD56+ HLA-DR+	- 14.4	(5.1 – 24.1)	- 4.3	(2.7 – 9.2)	**0.005**
CD3+CD16+	**2.2**	**(1.1 – 3.8)**	**1.8**	**(1.1 – 3.6)**	0.7
Contaminants / Undefined	**3.7**	**(3.0 – 5.2)**	**2.4**	**(1.9 – 3.9)**	**0.013**

* After exclusion of MAIT and γ/δ T-cells. Values expressed as percentage of parent population, Median (interquartile range). Significance of between group differences determined by Mann-Whitney U test.

Table 4-3 Lymphocyte sub-set phenotypes in CF subjects based on the presence of *P. aeruginosa* infection compared to healthy control subjects

	CF, No *P. aeruginosa* (n=5)	CF, *P. aeruginosa* infection (n=36)	P-Value^	Non-CF (n=22)	P-Value$
Sex (Female:Male)	3:2	14:22	0.4	11:11	1.0
Age (years)	23.3 (18.4-41.7)	28 (24.5-32.6)	0.8	26 (25 - 32)	0.5
BMI (kg/metre^2)	24.7 (19.9-31.7)	23.4 (20.5-25.3)	0.5	24.6 (21.4 – 28.3)	0.8
FEV_1 % Predicted (%)	72.4 (59.1-88.8)	63.6 (43.4-80.1)	0.3	110 (101 – 120)	**0.001**
FVC % Predicted (%)	83.9 (68.1-95.9)	79.3 (71.1-88.7)	0.5	107 (95 – 112)	**0.012**
Lymphocyte Population (size gated, CD14-)					
T-Cells (CD3+CD16-)	**68.2 (63.4 – 76.8)**	**72.9 (68.3 – 81.3)**	0.4	**74.9 (67.9 – 79.0)**	0.3
- CD4+CD8-	- 64.4 (49.6 – 70.0)	- 65.2 (56.9 – 70.5)	0.6	- 65.1 (59.9 – 71.4)	0.5
- CD8+CD4-	- 26.2 (22.8 – 38.8)	- 25.1 (20.5 – 33.1)	0.6	- 27.4 (22.7 – 32.8)	0.9
- MAIT Cells (CD161+, TCR Va7.2+)	- 2.4 (1.4 – 3.6)	- 1.0 (0.3 – 1.8)	**0.023**	- 2.0 (1.4 – 3.1)	1.0
- γ/δ T-cells (TCR γ/δ+)	- 8.0 (6.0 – 16.0)	- 10.5 (6.6 – 13.4)	0.7	- 6.4 (4.6 – 9.4)	0.2
- CD4+CD8+	- 0.2 (0.2 – 0.2)	- 0.3 (0.2 – 1.1)	**0.014**	- 0.4 (0.3 – 1.7)	**0.004**
- CD4-CD8-*	- 3.1 (1.6 – 4.1)	- 2.1 (0.7 – 2.8)	0.2	- 1.6 (1.1 – 2.3)	0.1
B-Cells (CD3-CD16-CD20+HLA-DR+)	**17.2 (14.0 – 20.9)**	**8.5 (3.6 – 13.0)**	**0.002**	**4.8 (3.9 – 8.7)**	**0.003**
- Non-Resting (CD1c-)	- 74.5 (69.1 – 79.0)	- 64.1 (55.5 – 73.7)	0.1	- 71.6 (60.5 – 75.1)	0.1
- Resting (CD1c+)	- 24.9 (20.3 – 30.5)	- 35.9 (25.3 – 43.9)	0.1	- 28.1 (25.0 – 38.3)	0.1
NK-Cells (CD3-)	**9.6 (4.6 – 10.9)**	**9.5 (7.8 – 12.5)**	**0.5**	**13.1 (8.0 – 18.0)**	**0.039**
- CD16+ CD56dim HLA-DR-	- 69.2 (48.5 – 80.9)	- 76.1 (63.8 – 88.1)	0.2	- 88.0 (84.7 – 92.6)	**0.006**
- CD16- CD56+ HLA-DR-	- 10.9 (7.1 – 14.5)	- 8.5 (6.1 – 13.3)	0.5	- 6.1 (4.5 – 9.1)	**0.1**
- CD16- CD56+ HLA-DR+	- 21.4 (10.8 – 37.4)	- 14.2 (4.5 – 23.8)	0.2	- 4.3 (2.7 – 9.2)	**0.006**
CD3+CD16+	**2.0 (0.9 – 2.6)**	**2.5 (1.0 – 4.2)**	0.4	**1.8 (1.1 – 3.6)**	0.9
Contaminants / Undefined	**1.8 (1.6 – 3.5)**	**3.7 (3.1 – 5.4)**	0.017	**2.4 (1.9 – 3.9)**	**0.4**

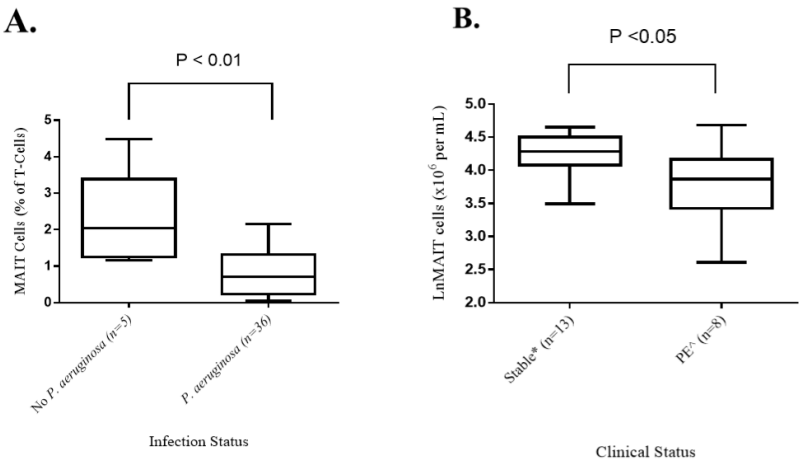
^CF No *P. aeruginosa versus* CF *P. aeruginosa* infection. $CF No *P. aeruginosa versus* Non-CF. *After exclusion of MAIT and γ/δ T-cells. Values expressed as percentage of parent population, Median (interquartile range). Significance of between group differences determined by Mann-Whitney U test.

Table 4-4 Lymphocyte sub-sets in CF subjects based on clinical stability and compared to healthy control subjects

	CF, Pulmonary exacerbation (n=8)	CF, Stable (n=13)	P-Value^	Non-CF (n=22)	P-Value$
Sex (Female:Male)	1:7	8:5	**0.027**	11:11	0.5
Age (years)	32.2 (30.1-39.1)	22.7 (20.5-26.9)	**0.001**	26 (25 - 32)	**0.010**
BMI ($kg/metre^2$)	24.6 (23.5-26.0)	23.0 (21.5-27.0)	0.4	24.6 (21.4 – 28.3)	0.7
FEV_1 % Predicted (%)	49.7 (40.3-68.5)	83.2 (74.1-88.8)	**0.001**	110 (101 – 120)	**<0.001**
FVC % Predicted (%)	74.6 (71.2-79.3)	90.0 (85.9-96.4)	**0.001**	107 (95 – 112)	**0.001**
Lymphocyte Population (size gated, CD14-)					
T-Cells (CD3+CD16-)	**71.6 (68.1 – 82.3)**	**71.3 (67.1 – 77.1)**	0.6	**74.9 (67.9 – 79.0)**	0.3
- CD4+CD8-	- 65.9 (52.2 – 76.2)	- 58.3 (55.3 – 68.9)	0.5	- 65.1 (59.9 – 71.4)	0.1
- CD8+CD4-	- 23.8 (16.4 – 37.8)	- 29.2 (23.6 – 35.3)	0.3	- 27.4 (22.7 – 32.8)	0.7
- MAIT Cells (CD161+, TCR Va7.2+)	- 1.1 (0.3 – 2.1)	- 1.1 (0.9 – 1.9)	0.7	- 2.0 (1.4 – 3.1)	**0.018**
- γ/δ T-cells (TCR γ/δ+)	- 11.1 (7.1 – 16.5)	- 10.5 (6.4 – 13.6)	0.8	- 6.4 (4.6 – 9.4)	**0.017**
- CD4+CD8+	- 0.2 (0.2 – 1.0)	- 0.2 (0.2 – 0.3)	0.5	- 0.4 (0.3 – 1.7)	**0.010**
- CD4-CD8-*	- 2.6 (1.4 – 3.5)	- 2.2 (1.3 – 3.4)	0.6	- 1.6 (1.1 – 2.3)	0.1
B-Cells (CD3-CD16-CD20+HLA-DR+)	**9.6 (2.5 – 14.0)**	**13.1 (8.5 – 16.7)**	0.3	**4.8 (3.9 – 8.7)**	**0.001**
- Non-Resting (CD1c-)	- 73.4 (65.5 – 79.9)	- 70.4 (59.9 – 79.9)	0.8	- 71.6 (60.5 – 75.1)	0.7
- Resting (CD1c+)	- 25.6 (19.9 – 34.1)	- 29.3 (18.1 – 40.1)	0.8	- 28.1 (25.0 – 38.3)	0.7
NK-Cells (CD3-)	**10.1 (5.9 – 12.6)**	**9.6 (5.4 – 11.8)**	0.7	**13.1 (8.0 – 18.0)**	**0.020**
- CD16+ CD56dim HLA-DR-	- 70.7 (52.9 – 83.7)	- 69.2 (55.3 – 80.5)	0.9	- 88.0 (84.7 – 92.6)	**<0.001**
- CD16- CD56+ HLA-DR-	- 9.9 (8.6 – 12.6)	- 8.0 (6.7 – 13.3)	0.6	- 6.1 (4.5 – 9.1)	**0.026**
- CD16- CD56+ HLA-DR+	- 19.4 (5.8 – 37.2)	- 21.4 (13.2 – 30.6)	0.8	- 4.3 (2.7 – 9.2)	**<0.001**
CD3+CD16+	**2.0 (1.2 – 4.0)**	**2.5 (1.1 – 4.2)**	1.0	**1.8 (1.1 – 3.6)**	0.7
Contaminants / Undefined	**3.6 (3.1 – 4.2)**	**3.5 (2.5 – 4.1)**	0.6	**2.4 (1.9 – 3.9)**	**0.4**

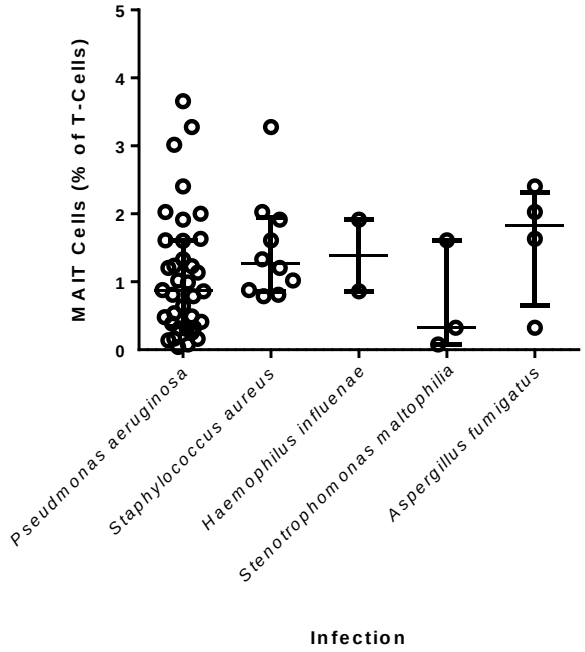
^ CF pulmonary exacerbation *versus* CF stable, $CF stable *versus* Non-CF. *After exclusion of MAIT and γ/δ T-cells. Values expressed as percentage of parent population, Median (interquartile range). Significance of between group differences determined by Mann-Whitney U test.

Figure 4-4 MAIT Cell percentage in CF subjects based on A. Presence of *P. aeruginosa* in sputum cultures, B. Clinical status.



PE: pulmonary exacerbations, *two and ^one not infected with *P. aeruginosa*, between group differences determined by Mann-Whitney U test.

Figure 4-5 MAIT cell percentage in CF subjects with *Pseudomonas aeruginosa* and a co-pathogen in sputum culture.



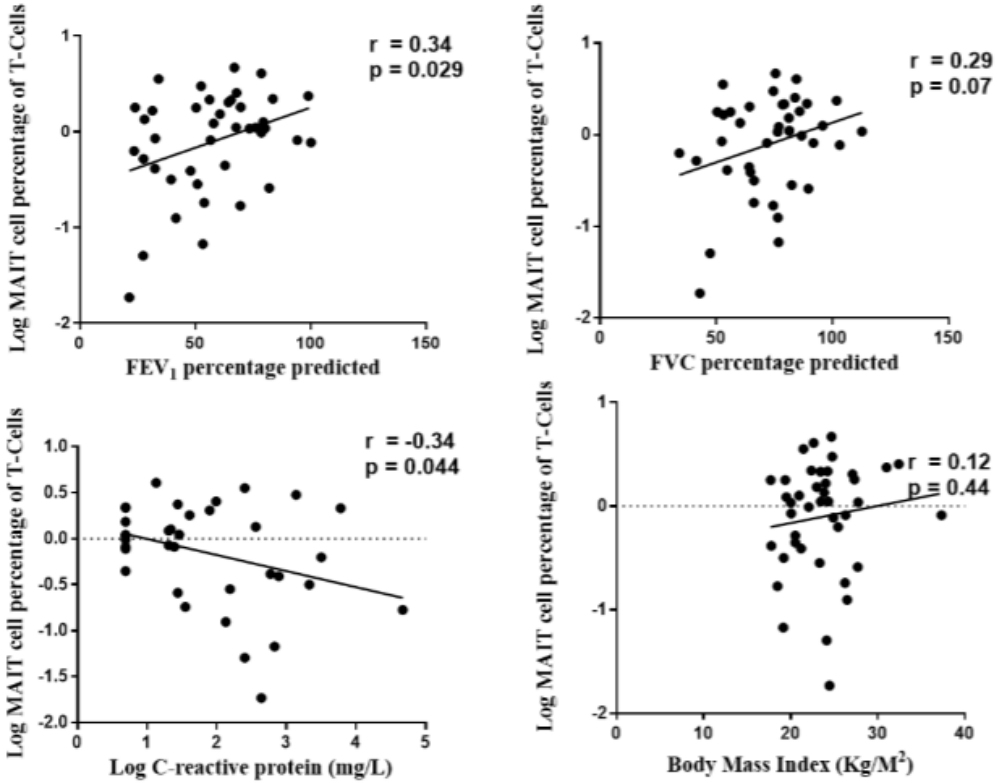
Individual subjects may be represented multiple times, dependent on the number of co-pathogens isolated from sputum cultures. Horizontal lines represent Median and interquartile range. No significant difference between groups when examined by Kruskal-Wallis test.

Figure 4-6 Relationship between MAIT and γ/δ T-cell counts and percentages with lung function.

A.

	LnMAIT Cell Concentration (cells per mL)	γ/δ T-Cell Concentration (cells per mL)
FEV_1% predicted	r = 0.47 p = 0.003	r = 0.35 p = 0.030
FEV_1% predicted decline per year	r = 0.28 p = 0.10	r = 0.31 p = 0.06
FVC% predicted	r = 0.43 p = 0.006	r = 0.33 p = 0.042
Body Mass Index (kg/m^2)	r = 0.12 P = 0.48	r = -0.03 P = 0.86
C-reactive protein (mg/L)	r = -0.39 p = 0.021	r = -0.44 p = 0.008

B.



A. Pearson's Correlation co-efficient (r) and significance value (p) of MAIT and γ/δ T-cells and B. Correlation plots for MAIT Cell expressed as percentage of T-cell population, with FEV_1 and FVC % predicted, C-reactive protein and body mass index. MAIT: Mucosal invariant T-Lymphocytes, γ/δ T-Cell: Gamma-Delta T-lymphocytes, FEV_1: Forced expiratory volume in one second, FVC: Forced vital capacity.

Absolute MAIT cell count and MAIT cell percentage correlated positively with FEV_1 and FVC percentage predicted. A weak relationship was seen between increased rate of FEV_1 decline and MAIT cells counts, but this did not reach statistical significance (Figure 4.6).
MAIT cell percentage and absolute MAIT cell concentrations were inversely correlated with CRP (r= -0.34, p= 0.044 and r= -0.39, p= 0.021, respectively), however, there was no relationship between MAIT cells and total WCC.

4.4.2 Relationship between γ/δ T-cells, microbiological and clinical parameters in subjects with CF.

Absolute γ/δ T-cell counts correlated with FEV_1 and FVC percentage predicted values. A trend toward more rapid rate of lung function decline in FEV_1 was seen in subjects with lower γ/δ T-cells numbers (Figure 4.6A). No relationship between γ/δ T-cells when expressed as a percentage of total T-cells and lung function parameters was observed.
γ/δ T-cell percentage and absolute cell number were inversely correlated with CRP (r= -0.44, p=0.008) and absolute γ/δ T-cell number was higher in stable CF subjects compared to subjects experiencing a pulmonary exacerbation (Figure 4.7).
There was no relationship between *γ/δ* T-cell counts or percentages and absolute WCC or profile of infection with *P. aeruginosa* (Table 4.3).

4.4.3 Relationship between other lymphocyte sub-subset and clinical parameter.

Absolute blood lymphocyte count was positively correlated with FEV_1 and FVC (litres and percentage predicted), however, no relationship was seen between absolute WCC and the percentage of the major lymphocyte sub-sets (T-cells, B-cells, NK-cells, CD3+/CD16+ cells) or lung function (Table 4.5).

Total lymphocyte count was decreased in CF subjects being treated for a pulmonary exacerbation.

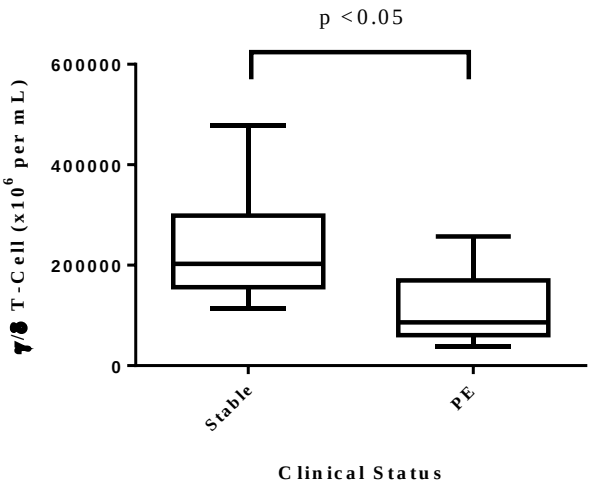
B-cell percentage was higher in subjects not infected with *P. aeruginosa*, compared to CF subjects with chronic *P. aeruginosa* infection and healthy controls. There was a trend towards lower B-cell percentages in CF subjects during a pulmonary exacerbation compared to stable CF patients (Figure 4.8A and B).

Table 4-5 Relationship between White cell count and lymphocyte subsets, and C-reactive protein, body mass index and lung function.

	FEV1 % Predicted	FVC % Predicted	BMI (Kg/m^2)	CRP (mg/L)
White Cell Count* ($x10^6$ per mL)	r = 0.17 p = 0.30	r = 0.18 p = 0.28	r = -0.14 p = 0.38	r = -0.02 p = 0.92
Lymphocyte ($x10^6$ per mL)	**r = 0.50** **p = 0.001**	**r = 0.49** **p = 0.002**	r = -0.07 p = 0.69	**r = -0.45** **p = 0.007**
T-cell (% of lymphocytes)	r = -0.12 p = 0.48	r = -0.11 p = 0.50	r = -0.15 p = 0.0.36	r = 0.28 p = 0.10
B-Cell (% of lymphocytes)	r = 0.27 p = 0.09	r = 0.17 p = 0.30	**r = 0.34** **p = 0.031**	r = -0.17 p = 0.31
NKT-Cell* (% of lymphocytes)	r = 0.08 p = 0.61	r = 0.13 p = 0.43	r = -0.14 p = 0.48	r = -0.12 p = 0.48
NK-Cell* (% of lymphocytes)	r = -0.07 p = 0.68	r = -0.04 p = 0.79	r = -0.10 p = 0.53	r = -0.25 p = 0.15
CRP* (mg/L)	**r = -0.54** **p = 0.001**	**r = -0.51** **p = 0.002**	r = -0.22 p = 0.20	

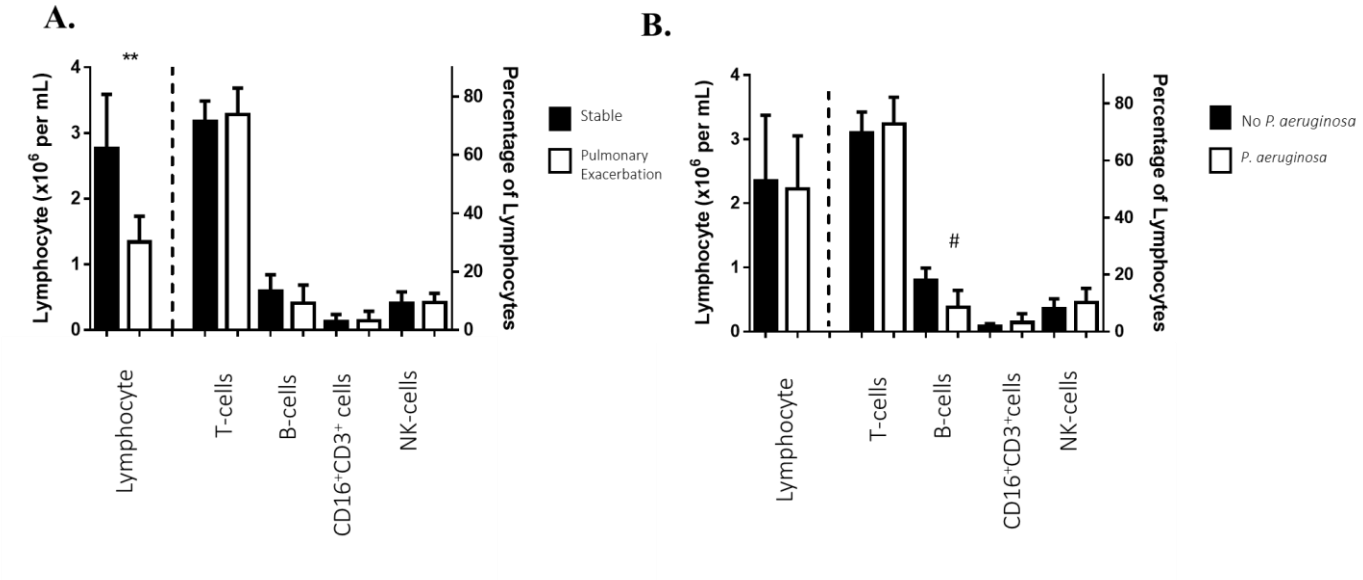
FEV1: Forced expiratory volume for one second, FVC: Forced vital capacity. *Ln Transformed prior to analysis. Pearson's correlations (r) and p-value.

Figure 4-7 Comparison of γ/δ T-cell counts in stable subjects and subjects undergoing antibiotic treatment for a pulmonary exacerbation.



PE: pulmonary exacerbations. Between group differences determined by Mann-Whitney U test.

Figure 4-8 Comparison of lymphocyte counts and percentage of lymphocyte sub-sets between A. Stable and pulmonary exacerbations B. *P. aeruginosa* infected and non-infected CF subjects.



Between group differences examined by Mann-Whitney U test. ** P=0.001 # P=0.004

4.5 Discussion.

In this study we demonstrate for the first time that the peripheral blood of subjects with CF is characterized by a relative lymphopenia and major reductions in circulating MAIT cells and to a lesser extent an increase in γ/δ T-cells compared to normal healthy controls, consistent with both quantitative and qualitative differences in innate T-cell immunity in CF. Importantly, lung disease severity, systemic inflammation, clinical status and the presence or absence of chronic *P. aeruginosa* infection were all significantly related to the number of circulating MAIT cells in peripheral blood.

The reduction in absolute lymphocyte counts as lung disease severity increases in the current study is consistent with a single previous study of peripheral blood lymphocytes in children with CF(276). Similar to this earlier study, a reduction in the percentage of NK-cells was also seen in subjects with CF, however, in contrast, no difference in the percentage of CD4+ T cells was seen and the percentages of other major lymphocyte subsets were similar between CF subjects and healthy control subjects(276). NK-cells represent an innate, cytotoxic subset of lymphocyte which primarily respond to viral infections and tumour cells(279). In addition, NK cells provide critical support to the eradication of bacterial pulmonary infection (including *P. aeruginosa*), principally through the generation of Th2 cytokines and IFN-γ(280, 281).

A higher percentage of B-cells was seen in CF subjects not infected with *P. aeruginosa* and a trend towards lower concentrations in subjects who were undergoing treatment for an acute pulmonary exacerbation. B-cells are critical for pulmonary protection against encapsulated bacteria and B-cells cultured *in vitro* respond to the mucoid exopolysaccharide of *P. aeruginosa*(282). Further longitudinal studies are required to delineate the role of B-cells during acute pulmonary exacerbations and to determine whether changes in circulating numbers can be attributed to peripheral destruction, tissue sequestration or transformation to plasma cells.

The association between MAIT cells counts and *P. aeruginosa* infection, severe lung function impairment, increased systemic inflammation and acute pulmonary exacerbations in subjects with CF may suggest that MAIT cell deficiency is associated with susceptibility to pulmonary infection in CF. However, the sub-group of CF subjects undergoing a pulmonary

exacerbation may have been biased towards a lower MAIT cell concentration, as more subjects in this sub-group were male and in general they had poorer baseline lung function(283). Furthermore, these data are cross-sectional and do not inform on whether low MAIT cell numbers contribute to *P. aeruginosa* colonisation and disease progression, or whether low numbers simply reflect depletion of MAIT cells in the circulation, because of recruitment to the airway mucosa.

MAIT cells provide a pivotal link between the innate and adaptive immune responses. The semi-invariant T-cell receptor on MAIT cells recognises metabolite derivatives of pathogens (e.g. vitamin B (riboflavin and folic acid)) presented combined to MR-1(284). Activated MAIT cells produce high concentrations of pro-inflammatory interleukin (IL)-17(220), which invokes a potent cascade of cytokines and chemokines (e.g. IL-8 and G-CSF) that promote neutrophil migration into the airways. Interleukin-17 is involved in neutrophil recruitment in CF and high concentrations have been described in sputum and bronchoalveolar lavage fluid of subjects with CF infected with *P. aeruginosa* and also in those patients with severe disease(55, 56, 285).

Mouse models demonstrate MAIT cell recruitment into the lungs at a very early stage of bacterial infection and sustained MAIT cell responsiveness during the late stages of infection contributes towards an ongoing cytokine response(72). In mice with selective MAIT cell deficiency the immune response was ineffective in controlling pulmonary infection(72).

Whilst we provide the first description of circulating MAIT cells in CF, understanding of the role of MAIT cells in human disease is limited. A reduction in the proportion of circulating MAIT cells has been reported in subjects with human immunodeficiency virus (HIV), *Mycobacterium tuberculosis* (MTB) and other severe pulmonary infections, and sepsis(69, 70, 286-288). In HIV infection, blood MAIT cells concentrations decrease progressively from time of infection, however, MAIT cell density remains relatively preserved in rectal mucosa, suggesting possible preferential recruitment and thus loss from the circulating pool(289, 290). Similarly, MTB reactive MAIT cells are enriched in lung lymph of healthy people when compared to matched blood samples(291). Collectively, the results of these earlier studies and our current work suggest that although blood MAIT cell counts may not reflect tissue concentrations, they may still prove to be a useful, surrogate biomarker of the immune response within the lung(292). We demonstrate a strong, inverse relationship between MAIT

cell counts and CRP and the specificity of MAIT cell responsiveness to bacterial and fungal infections, offers the potential for improved specificity compared to CRP, which will respond to both viruses and non-infective sources of systemic inflammation(293). Further mechanistic studies of MAIT cells in CF, including assessment in the actual airway will provide novel insights into their role in innate and adaptive immunity in the CF lung, including whether effective vaccines can be developed that boost MAIT cell function to allow eradication of key bacterial pathogens.

γ/δ T-cells represent a minor population of circulating T-cells, which has the capacity to expand rapidly in response to bacterial infection(66). γ/δ T-and MAIT cells share many similarities; they both produce IL-17 and preferentially migrate to mucosal surfaces from the circulation(67). In murine pulmonary infection models, γ/δ T-cells rapidly accumulate in the lung, in response to a range bacterial pathogens, where they facilitate the influx of neutrophils and subsequent bacterial clearance(66). The small increase in the proportion of γ/δ T-cells in the blood of CF subjects in the current study is consistent with findings of a single previous study in CF(68). However, the relationship between γ/δ T-cells and clinical parameters in CF subjects were only significant when absolute γ/δ T-cell concentrations were considered and these relationships may simply reflect changes in the absolute numbers of T-cells, rather than any implying any specific role for γ/δ T-cell in pathology.

Our current study has several limitations. Firstly, cross-sectional data collection, does not inform whether a causal relationships exists between lymphocytes subsets and clinical parameters. Furthermore, the limited number of patients included in the analysis of stable disease *versus* pulmonary exacerbation, and inclusion of only five patients not infected with *P. aeruginosa* means type I statistical errors are possible. Longitudinal studies which collect blood samples from the same subject before and after the acquisition of *P. aeruginosa,* or during a pulmonary exacerbation and again during a period of stable disease are required. Finally, blood lymphocyte population may not reflect airway populations and studies which correlate lymphocyte concentrations in airway biopsies to those in the blood are anticipated.

4.6 Conclusion.

In summary, we describe important differences in the proportions of circulating MAIT and γ/δ T-cell in adult patients with CF, compared to healthy control subjects. Reduced numbers

of MAIT cells were associated with *P. aeruginosa* pulmonary infection and more severe lung disease. Our findings provide the impetus for future studies examining the utility of MAIT cells in immunotherapies and vaccine development, and longitudinal studies of MAIT cells as biomarkers of CF pulmonary infection.

Chapter 5. The effects of bio-active metals in the CF airway on disease severity.

5.1 Abstract.

Background: Bio-active trace metals have been identified in respiratory tract secretions of subjects with lung disease and may potentially influence bacterial virulence, inflammation and disease severity. We measured a diverse range of metal ions in sputum samples from subjects with CF and non-CF bronchiectasis (NCFB) compared to healthy controls and examined their relationship to airway inflammation, disease severity and the presence of bacterial pathogens.

Methods: We studied 45 subjects with CF, 8 with NCFB and 8 healthy controls. Metal concentrations were measured in sputum supernatant by inductively-coupled plasma mass spectrometry and correlated with sputum inflammatory cell counts, lactate dehydrogenase (LDH) and interleukin (IL)-8 concentrations, lung function, clinical status and participant demographics.

Results: Sputum from subjects with CF and NCFB contained increased concentrations of magnesium, calcium, iron and zinc. Metal ion concentrations correlated positively with LDH levels. The concentrations of magnesium, iron and zinc positively correlated with IL-8. A sub-group of CF subjects with severe lung disease demonstrated increased sputum molybdenum concentrations.

Conclusion: Elevated concentrations of sputum metal ions appear to be associated with cell/tissue necrosis and inflammation in subjects with CF and NCFB. Sputum molybdenum concentrations may be a biomarker of severe CF airways disease.

5.2 Introduction.

The majority of individuals with cystic fibrosis (CF), the most common lethal genetic condition in Caucasians, will die prematurely from complications of chronic pulmonary sepsis(127). Intermittent airway infection and inflammation in CF begins in infancy, resulting in early onset bronchiectasis in many children(294). By adulthood, chronic, poly-microbial infection is established, in which *Pseudomonas aeruginosa* is the dominant pathogen in more than eighty percent of patients(1). The host immune response to infection in CF is intense and persistent, but is ineffective in clearing bacterial infection from the airways and contributes to local tissue destruction through the generation of proteolytic enzymes and reactive oxygen species (ROS)(295).

As pulmonary disease progresses, plugging of the distal airways by dehydrated sputum, creates micro-aerobic or frankly anaerobic pockets, with low pH and altered nutrient availability(212, 213). The highly abnormal environment within these regions drives phenotypic adaptation and alters the virulence of the incumbent bacteria, favouring pathogens capable of survival in low oxygen environments(213).

Bio-active trace metals (biometals) are essential co-factors in a wide range of human and bacterial enzyme systems, however, strict regulation of their bioavailability is essential to prevent toxicity(215). A limited number of previous studies in patients with CF, non-CF bronchiectasis and chronic bronchitis have demonstrated increased iron (Fe), zinc (Zn) and copper (Cu) concentrations in airway secretions and have postulated that these metals may influence disease severity(179, 216).

There is increasing interest in Fe in the CF lung. *In vitro* studies demonstrate that Fe strongly influences the ability of *P. aeruginosa* to form co-dependent bacterial communities (biofilms), which represent the major barrier to the eradication of established airway infection. Manipulation of Fe availability has therefore been proposed as a novel therapeutic strategy for the treatment of chronic *P. aeruginosa* infection(196, 217). An observational study by Gray and colleagues has previously demonstrated increased concentrations of Zn and Cu in the CF lung(216), and in separate *in vitro* studies these metals have been shown to induce *P. aeruginosa* resistance to carbapenem antibiotics(218). From the host perspective, airway Fe and other redox active biometals may catalyse the production of ROS and promote

inflammation(214). Conversely, Cu and Zn are key components of airway anti-inflammatory superoxide dismutases (SODs)(296). The origin of airway metal ions has not been determined, with potential sources including vascular leak, channelopathies and release from necrotic airway cells, or the bacteria themselves.

In this study, we expand on previous work by examining a more diverse range of biometals in expectorated sputum samples from patients with CF and non-CF bronchiectasis (NCFB). Sputum biometal concentrations are compared to those in healthy controls, and correlated with sputum lactate dehydrogenase (LDH) as an index of local tissue necrosis and interleukin (IL)-8 as a marker of airway inflammation and host immune response. To examine the influence of infection and clinical status on biometal concentration, we included a sub-group of patients with CF who were not infected with *P. aeruginosa* and followed a group of *P. aeruginosa* infected patients through treatment of a pulmonary exacerbation with a course of intravenous antibiotic therapy.

Calcium (Ca), magnesium (Mg), manganese (Mn), Zn and Cu were selected for their importance in inflammatory pathways(214, 296, 297). Molybdenum and Fe were selected for their potential importance to bacterial virulence and anaerobic respiration(179, 298). Finally, nickel (Ni) and lead (Pb) were included as potential indicators of environmental contamination(299, 300).

5.3 Methods.

5.3.1 Participants and sample collection.

Participants were recruited from The Prince Charles Hospital, Queensland and Royal Hobart Hospital, Tasmania, Australia. Institutional human research and ethics committee approval was gained from both sites (HREC2008:2885 and H0009813 respectively). Sixty-seven spontaneously expectorated sputum sample were collected from 45 subjects with CF and a single sputum sample was collected from eight subjects with NCFB. Induced sputum was collected from eight healthy controls following inhalation of nebulised 4.5% hypertonic saline. For cross-sectional analyses, the initial sputum sample collected from each subject was used. Eleven subjects with CF provided samples at several time-points during intravenous antibiotic treatment of a pulmonary exacerbation. Participant demographics,

including infecting pathogens were recorded (Table 5.1). None of the participants reported regular tobacco smoking.

5.3.2 Sputum processing.

Sputum plugs free of salivary contamination were separated from the expectorated sputum samples and homogenised with dithiothreitol as previously described(301). Homogenised samples were diluted with phosphate buffered saline (dilution factor 1:10) and centrifuged to pellet cells. The cell free supernatant was removed and stored at -80°C for later batch analysis of biometals. The cell pellet was resuspended and total inflammatory cell count (TCC) performed using standard methodology(301).

5.3.3 Inductively coupled plasma mass-spectrometry (ICPMS) analysis.

To prepare samples for ICP-MS, 500 μL of sputum supernatant was digested overnight in an acid-cleaned tube containing 500 μL of 3M double-distilled nitric acid (HNO_3) and 1 mL of an internal standard spike solution (^{6}He, ^{61}Ni, ^{103}Rh, ^{115}In, ^{187}Re and ^{209}Bi). Digested samples were diluted to 10 mL with 0.3M HNO_3 and analysed using a Thermo Fischer X Series ICPMS machine. Four replicates per sample were averaged for the final calculation. For calibration and to establish recovery rates, standard reference materials W2 and SLRS5 and multi-element standards were analysed at the beginning and the end of the run. Internal standards were used to correct for internal drift. A monitor solution, used to correct for external drift, and an instrument blank solution, used to monitor baseline drift and memory effects, were analysed at regular intervals throughout the run.

5.3.4 LDH and IL-8 measurement.

LDH activity was determined in thawed sputum supernatants warmed to 37°C using a LDH colorimetric assay kit (Abcam Inc, Cambridge, MA) according to the manufacturer's protocol. IL-8 (biolegend, USA) concentration was measured according to the manufacturer's protocol with a detection range between 31.25-4000 pg/ml. Each sample was diluted as necessary to fall in the linear range and assayed in duplicate.

5.3.5 Air quality assessment.

To examine whether atmospheric pollutants potentially contributed to sputum metal concentrations, we recorded the level of atmospheric particulate matter 10 micrometres or smaller (PM_{10}) as reported by the Queensland Department of Environment and Heritage Protection (http://www.ehp.qld.gov.au/air/data/search.php) for the city of Brisbane on the day a sputum sample was collected. These data were available for subjects sampled through the Prince Charles Hospital, but no similar data were available for Tasmanian participants.

5.3.6 Statistical analysis

Between-group differences in continuous variables were analysed by independent samples t-test, analysis of covariance, Mann-Whitney U or Kruskal-Wallis H test, depending on number of independent groups and normality of the data. Pearson's correlation was used to examine relationships between lung function, TCC, IL-8, LDH, PM_{10} and biometal concentrations. A p-value <0.05 was considered to represent statistical significance. Data analysis was performed using PASW, Version 18.0 (SPSS Inc. Chicago, IL, USA)

5.4 Results.

5.4.1 Cross-sectional comparisons

Sixty-one samples (8 normal, 8 NCFB, 45 CF) were included in the cross-sectional analysis. Sputum from subjects with CF and NCFB demonstrated a high TCC and contained significantly higher concentrations of Mg, Ca, Fe and Zn compared to healthy controls (Table 5.2).

Table 5-1 Participant Demographics

	Normal (n=8)	**CF (n=45)**	**Non-CF Bronchiectasis (n=8)**
Age (years)	57 (7)	29 (12)	60 (10)[b]
Sex (M:F)	6:2	28:17	4:4
FEV_1 % predicted	106 (10)	53 (23)[a]	33 (15)[c]
FVC % predicted	110 (16)	65 (21)[a]	61 (6)[c]
BMI	n/a	21 (3)[b]	25 (4)[d]
Airway pathogens (n, %)			
- *Pseudomonas aeruginosa*	-	33 (73%)	6 (75%)
- MSSA	-	16 (36%)	-
- *Aspergillus spp*	-	9 (20%)	2 (25%)
- MRSA	-	2 (4%)	-
- *Burkholderia spp*	-	3 (6%)	-
- *Haemophilus influenzae*	-	1 (2%)	1 (13%)

Values reported as mean (standard deviation) unless otherwise stated, M:F: Male:Female ratio, data unavailable for [a] one, [b] two, [c] three, [d] four subjects, MSSA: methicillin sensitive *Staphylococcus aureus*, MRSA: methicillin resistant *Staphylococcus aureus*, *spp*: species. BMI: Body mass index. n/a: not available.

Sputum metal concentrations were compared to published data on biometal concentration in normal serum, whole blood and pleural fluid (Table 5.2)(302-306). Sputum from subjectts with CF and NCFB contained higher metal concentrations than serum and pleural fluid (except for Cu). Sputum from healthy controls contained lower concentrations of biometals than serum, pleural fluid and whole blood (except for Pb which was higher than in serum and pleural fluid).

In patients with CF, two distinct cohorts of subjects were detected with and without significant concentrations of Mo (Figure 5.1). Subjects with "elevated" Mo concentrations had lower FEV_1% predicted, but also lower TCC (Table 5.4).

Table 5-2 Comparison of sputum inflammatory markers and biometals in healthy controls, CF and non-CF bronchiectasis

	Normal (n=8)	CF (n=45)	Bronchiectasis (n=8)	Pleural Fluid [21]	Serum [Reference]	Blood [Reference]
TCC (10^6 cell/ml)	0 (0-1)	12 (4-21)[g]	24 (12-35)[f]			
LDH (mU/ml)	39 (0-95)[b]	50 (1-247)[d]	13 (3-1657)[a]			
IL-8 (ng/ml)	28 (15-64)[b]	135 (84-279)[c,f]	103 (13-292)[a]			
Mg (mg/L)	4 (2–7)	30 (19-44)[g]	33 (27-39)[f]	18	22 [295]	
Ca (mg/L)	45 (28–58)	102 (76-123)[g]	124 (78-156)[f]	71	96 [295]	
Mn (µg/L)	5 (2-9)	6 (4-17)	6 (4-10)	1	1.0 [295]	12 [296]
Fe (µg/L)	0 (0-37)	797 (398-1292)[g]	1075 (862-1324)[f]	n/a	4690 [298]	476000 [296]
Ni (µg/L)	12 (1-60)	5 (0-23)	26 (2-99)	n/a	2.2 [29]	2.7 [296]
Zn (µg/L)	179 (103-597)	1285 (678-1811)[g]	537 (401-838)[c h]	283	474 [295]	5800 [296]
Cu (µg/L)	106 (55.3-196)	173 (128-257)	226 (130-314)	530	1189 [295]	830 [296]
Mo (µg/L)	0 (0)	0 (0-181)[e]	0 (0)[i]	1	1.2 [295]	0.9 [296]
Pb (µg/L)	9 (4-21)	3 (1-6)[e]	2 (1-5)[e]	6	1.3 [295]	26 [299]

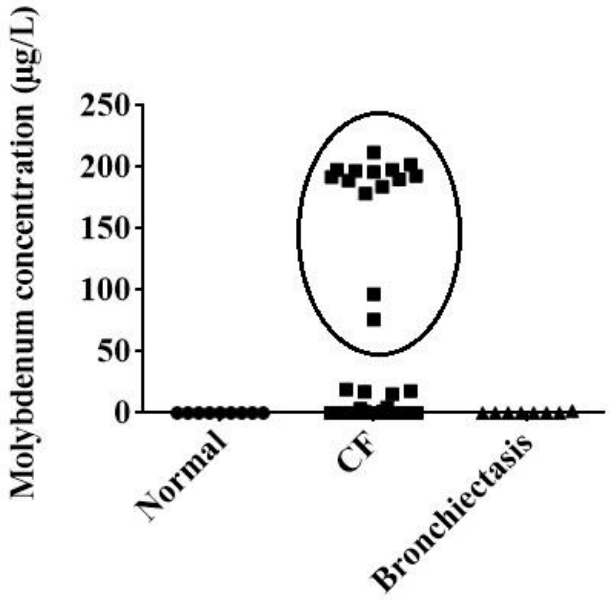
Values reported as median (interquartile range), data unavailable on [a] one patients, [b] three patients, [c] four patients, [d] six patients, mg/L: milligrams per litre, µL/L: micrograms per litres, ng/ml: nanograms per millilitre, significantly different to normal at [g] $p<0.001$, [f] $p<0.01$, [e] $p<0.05$ level, significantly different to CF at [i] $p<0.01$, [h] $p<0.05$ level (Mann Whitney U test).

Table 5-3 Correlation co-efficients of biometals in all sputum samples[c,d,e]

	Ca	Mn	Fe	Zn	Cu	FEV_1 % Predicted	TCC	IL-8	LDH	PM_{10}
Mg	0.89[a]	0.13	0.79[a]	0.75[a]	0.42[a]	-0.20	0.05	0.42[a]	0.44[a]	0.05
Ca	-	0.15	0.80[a]	0.67[a]	0.49[a]	-0.16	-0.02	0.23	0.33[a]	0.11
Mn	-	-	0.29[a]	0.12	0.06	0.28[b]	-0.13	0.15	-0.14	-0.14
Fe	-	-	-	0.75[a]	0.47[a]	-0.11	-0.08	0.39[a]	0.40[a]	0.03
Zn	-	-	-	-	0.46[a]	-0.18	0.02	0.38[a]	0.39[a]	0.03
Ni	-	-	-	-	-	-0.30[b]	-0.01	0.03	0.11	0.03
Cu	-	-	-	-	-	-0.20	-0.15	0.23	0.32[a]	0.04
Mo	-	-	-	-	-	-0.38[a]	-0.31	0.18	0.02	-0.03
Pb	-	-	-	-	-	-0.08	-0.13	-0.19	-0.10	-0.03

Values: Pearsons correlation - r values, [a] $p<0.01$, [b] $p<0.05$. [c]Correlation of Ni, Mo and Pb with other metals were not significant and are not included. [d]Correlations with FEV_1 % predicted, TCC, IL-8 and LDH do not include healthy control data. [e]Correlations with PM_{10} are based on 43 samples obtained from subjects with CF recruited from Queensland, where comparative air-quality data was available. FEV_1 % predicted: percentage predicted forced expiratory volume in one second. TCC: total inflammatory cell count. IL: interleukin. LDH: lactate dehydrogenase. PM_{10}: Particulate matter 10 micrometres or smaller in diameter.

Figure 5-1 Concentration of molybdenum in sputum samples.



Distribution of sputum sample Molybdenum concentrations by disease type. Circle, represents CF patient samples which were classified as containing a high Molybdenum concentration in subsequent analyses (see Table 5-4)

Six subjects with NCFB and 33 with CF were infected with *P. aeruginosa*. CF subjects infected with *P. aeruginosa* had lower lung function (Median FEV_1 % predicted 38% v 71%, $p<0.01$) and higher sputum concentration of IL-8 (165 v 70 ng/ml, $p<0.01$), Ca (105 v 74 mg/L, $p<0.01$) and Mg (22 v 47 mg/L, $p<0.05$) compared to subjects without *P. aeruginosa* infection, however TCCs and levels of other measured metals were not significantly different (Table 5.5).

5.4.2 Relationship between biometals, LDH, IL-8 and PM_{10} within samples.

A correlation analysis, including all sputum samples demonstrated that the concentrations of Mg, Ca, Fe, Zn and Cu were all strongly correlated with one another (Table 5.3). Conversely, no relationship was seen between concentrations of Pb, Mo or Ni. In sputum samples from subjects with CF and NCFB, both LDH and IL-8 concentrations positively correlated with the concentration of Mg, Fe and Zn, and LDH alone correlated with Ca and Cu concentrations (Table 5.3).

Table 5-4 Comparison of CF patients with elevated and non-elevated molybdenum levels.

	Non-elevated Mo (n=31)	**Elevate Mo (n=14)**	**p-value**
Age (years)	27 (20-39)	29 (19-38)	0.90
Sex (M:F)	19:12	9:5	
BMI (kg m^{-2})	20 (19-24)	20 (17-22)	0.42
FEV1 (% predicted)	53 (37-78)	36 (28-58)	0.09
FVC (% predicted)	67 (50-88)	56 (41-71)	**0.04**
TCC (10^6 cell/mL)	15 (6-26)	5 (2-10)	**0.01**
LDH (mU/ml)	43 (1-131)	56 (1-680)	0.13
IL-8 (ng/L)	105 (81-278)	232 (126-298)	0.41
Mg (mg/L)	33 (19–47)	27 (18-32)	0.17
Ca (mg/L)	105 (77–136)	90 (74-108)	0.16
Mn (µg/L)	6 (4-19)	7 (3-16)	0.73
Fe (µg/L)	797 (476-1242)	852 (347-1517)	0.93
Ni (µg/L)	1 (0-21)	21 (10-33)	**0.01**
Zn (µg/L)	1021 (587-1377)	1794 (835-2149)	0.04
Cu (µg/L)	185 (131-253)	150 (117-269)	0.49
Pb (µg/L)	3 (1-6)	2 (0-7)	0.27

Non-elevated Mo: <20 µg/L, Elevated Mo >= 20 µg/L. Data given as median (interquartile Range). Significant difference assessed by Mann Whitney U test.

Table 5-5 Comparison of CF subjects, infected and non-infected with *Pseudomonas aeruginosa*.

	P. aeruginosa **Infected (n=33)**		***P. aeruginosa*** **Non-infected (n=12)**		**p-value**
Age (years)	29	(22-36)	19	(17-41)	0.10
Sex (M:F)	19:14		9:3		
BMI (kg m^{-2})	21	(20-22)	20	(19-25)	0.93
FEV1 (% predicted)	38	(27-58)	71	(54-91)	**<0.01**
FVC (% predicted)	55	(44-71)	78	(62-99)	**<0.01**
TCC (10^6 cell/mL)	11	(5-19)	17	(4-55)	0.30
LDH (mU/ml)	54	(3-270)	2	(0-110)	0.10
IL-8 (ng/L)	165	(86-338)	70	(70-90)	**<0.01**
Mg (mg/L)	31	(22–47)	19	(13-33)	**<0.05**
Ca (mg/L)	105	(87–132)	74	(74-108)	**<0.01**
Mn (µg/L)	6	(4-18)	6	(3-18)	0.62
Fe (µg/L)	857	(460-1292)	649	(304-1271)	0.29
Ni (µg/L)	9	(0-23)	2	(0-21)	0.73
Zn (µg/L)	1304	(678-1693)	983	(635-2279)	0.88
Cu (µg/L)	185	(143-288)	130	(94-183)	0.05
Mo (µg/L)	0	(0-187)	1	(0-92	0.70
Pb (µg/L)	3	(0-7)	3	(1-6)	0.40

Data given as median (interquartile Range). Significant difference assessed by Mann Whitney U test.

Comparative PM_{10} data was available for 43 sputum samples from 23 CF subjects. There was no correlation between PM_{10} and metal concentrations in sputa on the day of collection (Table 5.3).

5.4.3 Sputum Biometals during pulmonary exacerbations.

Four CF subjects had increased sputum Pb levels on admission, which fell to trace levels during the course of the admission. Sputum Fe concentration increased during treatment in three subjects, of which, two received intercurrent iron infusions to correct severe iron deficiency. There was no consistent change in sputum concentrations of the other metals during treatment of a pulmonary exacerbation (Figure 5.2).

Figure 5-2 Longitudinal changes in sputum biometals for subjects with CF undergoing treatment for a pulmonary exacerbation.

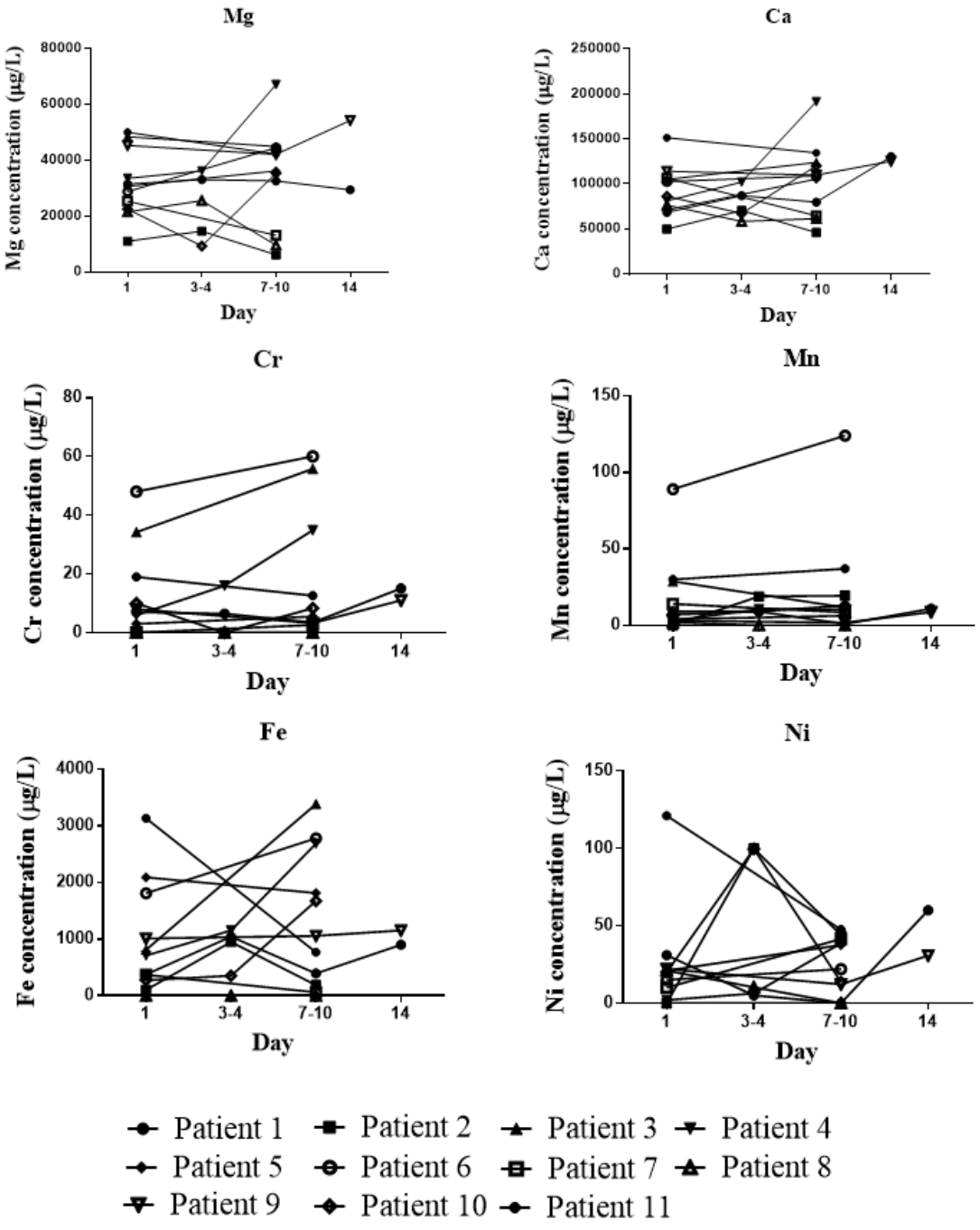
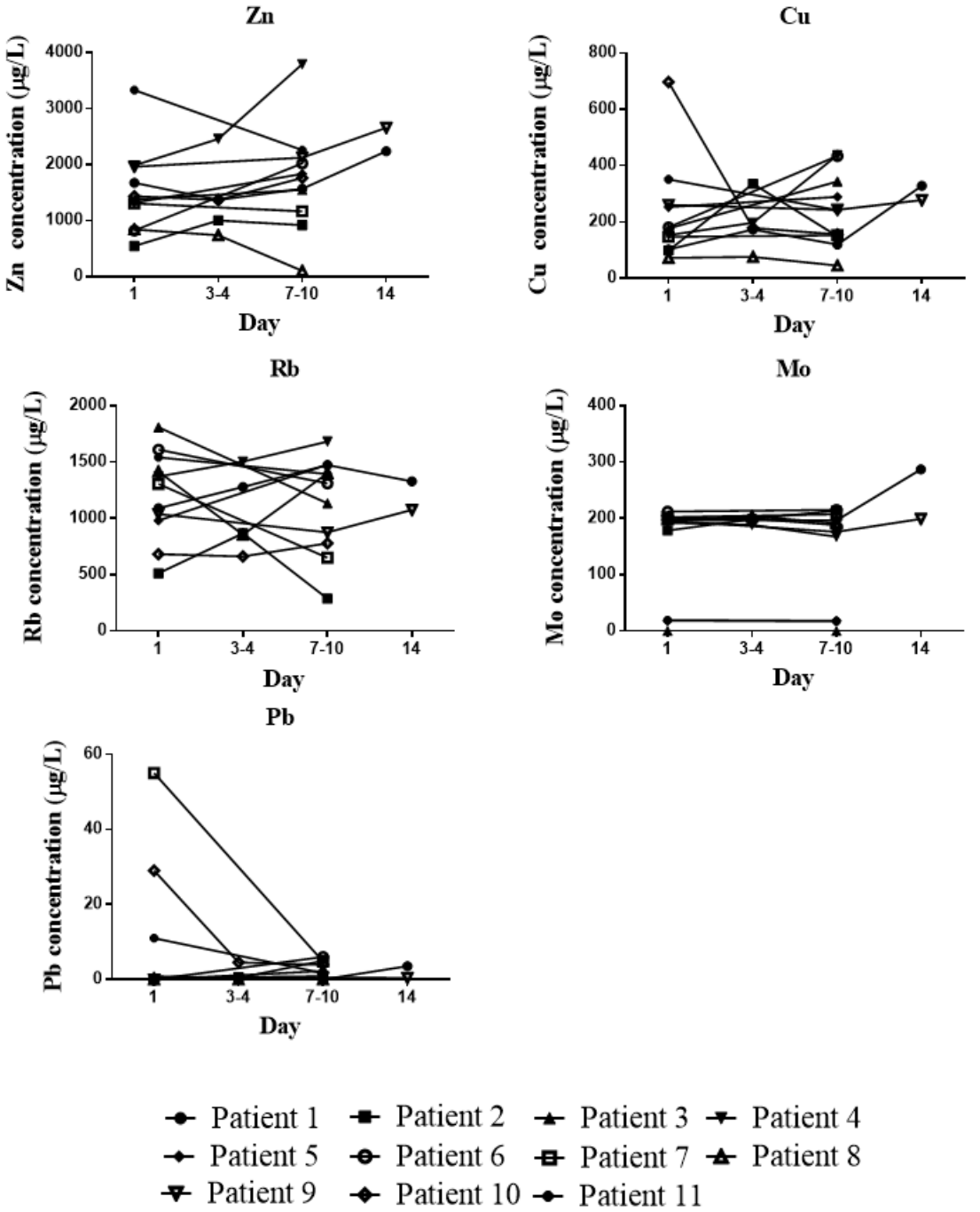


Figure 5.2 continued Longitudinal changes in sputum biometals for subjects with CF undergoing treatment for a pulmonary exacerbation.



5.5 Discussion.

This work provides important new insights into the inflammatory milieu present in the lungs of patients with CF and NCFB. A diverse range of biometals were present in the sputum of subjects with these suppurative lung diseases, several of which have the potential to significantly influence both host immunity and bacterial virulence(307).

Defining the importance of the biometals we detected in sputum and how these metals relate to lung disease severity will advance our understanding of the lung micro-environment in diseases characterised by chronic bacterial infection. Accurately characterising the presence of biometals, will also provide information that will be relevant to the development of *in vitro* infection models and potentially, identify targets for novel therapies(196).

The strong positive correlation between the concentrations of Ca, Mg, Fe, Zn and Cu suggests that these metals originate from a common source, or reflect a common disease process. LDH has previously been identified as a surrogate marker of pulmonary tissue damage and the positive correlation between several of the biometals detected and LDH levels supports cell injury as a probable contributor to the presence of some of these biometals(308). The concentration of sputum biometals more closely represent levels seen in whole blood compared to serum (Table 5.2) and several metals (Mg, Fe and Zn) correlated positively with the concentration of pro-inflammatory IL-8, adding further support to the hypothesis that release of these metals is associated with local tissue damage**.**

Consistent with previous studies, CF subjects infected with *P. aeruginosa* had worse lung function and higher sputum IL-8 concentration, than their non-infected counterparts(309). Surprisingly, there was a trend towards lower TCCs in the *P. aeruginosa* infected patients, however, sputum concentrations of Mg and Ca were significantly higher, which may again support a hypothesis that these metals are released from necrotic immune cells in the setting of heightened inflammation. From our results, it is not possible to determine whether the biometals originated from injured lung tissue or from luminal inflammatory cells and investigation of metal levels within the airway wall or lung parenchyma are required to address this question.

Other potential sources of biometals in sputum should be considered, including micro-vascular leakage, cell membrane channelopathies and contamination from inhaled particulates. We have previously reported increased concentrations of micro-albumin in CF sputa, which may suggest

microvascular leakage is contributory(177), but sputum biometal levels exceeded those reported in serum, which argues against this being the sole explanation(303-305).

CFTR-mutant airway epithelial cells (AECs) appear to "leak" Fe *in vitro*, which in-turn promotes growth of *P. aeruginosa* on their apical cell surface membrane, and suggests specific channelopathies may contribute to airway metal accumulation(180). For example, divalent metal-ion transporter (DMT)-1 is important in airway Fe detoxification and, in DMT-1 deficient rats, Fe accumulates in the airways, resulting in increased oxidative stress and inflammation(310). This observation is important, as abnormal function of metal transport defects may be amenable to therapeutic manipulation to limit the availability of metals to pathogenic bacteria.

The contribution of inhaled particulate matter to sputum metal concentrations was assessed by measurement of sputum of Pb and Ni concentrations and correlation of sputum biometal concentrations to atmospheric PM_{10} levels on the day of sample collection. Lead was detectable in the sputa of four CF subjects on admission to hospital, but not at subsequent time-points, raising the possibility that this metal was environmentally derived (e-figure 1). However, PM_{10} values did not correlate with sputum metal content in any of the subjects. Furthermore, Pb and Ni concentrations in subjects with CF and NCFB were not significantly different to those found in healthy controls. Taken together, these findings strongly suggest that environmental exposure to metals has minimal impact on sputum biometal content.

An important new finding is the identification of a distinct group of CF subjects with "elevated" concentrations of sputum Mo. Subjects with elevated Mo overall had more severe lung disease and a sub-group had CF related liver disease. Molybdenum is able to cycle between reduced (Mo^{4+}) and oxidised (Mo^{6+}) states under normal physiological conditions and facilitate the transfer of atomic oxygen between substrates in anoxic environments, which makes it essential to virtually all forms of life(298). In humans, Mo co-factored proteins are incorporated into the flavoenzymes, which catalyse critical detoxification pathways(311). Increased serum Mo concentrations have been reported in patients with liver disease, a finding not inconsistent with our sputum findings, although we did not assess Mo levels in serum to confirm a corresponding increase in systemic levels(312). In bacteria, Mo is an essential component of nitrate reductase, which catalyses the denitrification of nitrate during anaerobic respiration(298). Whilst *P. aeruginosa* is an aerobic bacterium, it can thrive under microaerobic and anaerobic conditions through the utilisation of nitrogen sources(313). Under anaerobic conditions, *P. aeruginosa* increases the production of Mo containing proteins and mutant strains that are unable to incorporate Mo have attenuated virulence(314). The evidence for

anaerobic bacterial respiration in the diseased CF lung is compelling, with the presence of a variety of obligate anaerobes identified in addition to *P. aeruginosa*(74, 313). It is biologically plausible that airway Mo may promote anaerobic respiration in resident bacteria, but this requires further investigation. Future, longitudinal studies, which correlate Mo concentrations with, clinical status (stable *versus* pulmonary exacerbation), lung function decline, infecting pathogens and serum Mo levels are required to determine its source and relevance to bacterial virulence and outcomes in patients with CF.

Increased Fe in the sputum of patients with CF has been demonstrated in several studies(176-179). Iron poses a risk to the lung through generation of toxic hydroxyl radicals and oxidative stress via Fenton chemistry and, as previously discussed, Fe has been shown to influence *P. aeruginosa* behaviour *in vitro*(217). Furthermore, Fe concentrations positively correlate with *P. aeruginosa* colony counts in the sputum of patients with CF confirming its importance *in vivo*(179).

Magnesium has generally been considered to be anti-inflammatory, but animal studies have shown that it may inhibit phagocytosis and impair the oxidative burst capacity of neutrophils, which will be detrimental in the setting of chronic infection(315). From a bacterial perspective, conflicting *in vitro* studies suggest Mg may inhibit formation of new *P. aeruginosa* biofilms, but protect established biofilms from disruption(316).

Calcium was also present in high concentrations in sputa from patients with CF and NCFB, and *in vitro* studies have demonstrated that Ca-dependent signalling enhances *P. aeruginosa* cell adherence, including adherence to AECs(317). Calcium increases *P. aeruginosa* biofilm thickness, and promotes the production of pyocyanin and other virulence factors(318). Pyocyanin itself, which is an important *P. aeruginosa* exotoxin, disturbs AEC Ca homeostasis, which will serve to further increase airway Ca concentrations(319).

The high concentration of Zn found in CF and, to a lesser extent, NCFB sputa is consistent with a single, previous publication. This finding is surprising given the high prevalence of systemic Zn deficiency reported in CF, and may therefore suggest selective Zn loss into the airways(300, 320). Human AEC strongly express the anti-oxidant Cu-Zn-SOD and systemic Zn deficiency increases airway inflammation and susceptibility to oxidative stress(296). Zinc co-factored metalloproteinases, such as ADAM33, contribute to airway remodelling in response to repeated insults(321). *In vitro* and animal models suggest Zn restores defective chloride transport by activating alternative Ca-dependent chloride channels and Zn supplementation has been shown to

be of modest benefit in reducing antibiotic requirements in children with CF(320). In bacteria, Zn is an essential metal, but also potentially toxic and its uptake is tightly regulated(218). *P. aeruginosa* Zn uptake is regulated by the operon CzcRS, which in the presence of Zn promotes expression of the metal efflux pump CzcCBA. Additionally, CzcRS down regulates OprD that confers resistance to carbapenem antibiotics, suggesting an increase in Zn within the airway may influence antibiotic susceptibility(218). The net effect on disease outcomes in CF and NCFB of increased airway Zn concentrations remains uncertain, given the anti-inflammatory properties *versus* potential promotion of antibiotic resistance, and requires further investigation.

This study has a number of limitations. Studies of airway pathology based on expectorated sputum are intrinsically associated with sampling variability and confounded by the possibility of oropharyngeal contamination, but we were very careful to select only airway plugs and salivary contamination with this practice is minimal(322). Only a small numbers of healthy controls and patients with NCFB, but our numbers are comparable with earlier reports(176, 178, 216). There was no change in sputum metal ion concentrations during treatment of a pulmonary exacerbation, suggesting that the measurement of metals has limited potential as a biomarker of therapeutic response. However, patients who provided serial samples were at the severe end of the disease spectrum. These individuals often respond sub-optimally to antibiotic treatment, in terms of their clinical improvement, reduction in bacterial numbers and inflammation, which may have limited our ability to detect meaningful changes(322). Future studies should examine whether biometal concentrations fluctuate in response to treatment in patients with milder disease, and also at a much earlier time-point in the disease process, i.e. prior to the onset of infection. Additionally, studies in subjects at an earlier disease time-point may allow metal concentrations to be correlated with the presence of different inflammatory cell types (i.e neutrophils *versus* macrophages), which is not possible in the adult subjects, with severe disease, where airway inflammation is dominated by neutrophils. Finally, analysis of the impact of specific bacterial infections, e.g. MRSA and *Burkholderia* species, on biometal levels is limited by the study size.

5.6 Conclusion.

In summary, this is the most extensive analysis of sputum biometals in patients with CF and NCFB undertaken to date. The positive correlation between sputum LDH and biometal concentrations suggests that local cell damage is associated with airway metal content, however, whether local tissue injury is the source or a consequence of the presence of redox active biometals in the airway requires further investigation. A sub-group of CF patients with severe lung disease were identifiable

by the presence of increased Mo in their sputum. It is likely that the complex composition of the biometals milieu in the lung strongly influences the behaviour of both the host immune response as well as bacterial pathogens. Mechanistic studies are required to establish the source of the metals detected and to determine their relevance to oxidative stress, impaired immune function and the promotion of bacterial virulence. Novel therapeutic interventions that modulate the availability of these biometals may reduce airway infection and substantially impact on disease progression.

Chapter 6. *HFE* as a Gene Modifier in CF

6.1 Abstract

Aims: Genetic modifiers contribute to variable disease phenotype in cystic fibrosis (CF). We explore the association between mutations in the haemochromatosis (*HFE*) gene and disease severity in adults with CF.

Methods: *HFE* genotyping was performed in 163 adults with CF attending a single centre. Results were correlated with lung disease severity, prevalence of CF-related diabetes (CFRD) and history of meconium ileus (MI) or distal intestinal obstruction syndrome (DIOS).

Results: Subjects with the C282Y substitution in the HFE protein (C282Y mutation) had a lower FEV_1 percentage predicted (51% *versus* 66%, p=0.032) and accelerated rate of FEV_1 decline (-110mL *versus* -80mL per year respectively, p<0.001) compared to subjects with a normal *HFE* genotype. C282Y substitutions were associated with increased rates of CFRD (58% versus 33%, P=0.022) and MI or DIOS (38% versus 19%, p=0.045). H63D HFE substitutions were associated with a more rapid rate of decline in forced vital capacity and increased risk of MI or DIOS.

Conclusions: In subjects with CF, the C282Y HFE substitution was associated with worse lung function, and increased rates of CFRD and gastrointestinal complications. The H63D HFE substitution also impacted on disease phenotype, but to a lesser extent. The results support a role for *HFE* gene mutations as modifiers of CF phenotype.

6.2 Introduction

Cystic fibrosis (CF) is an autosomal recessive, multi-organ disease, which results from mutations of the CF transmembrane conductance regulator (*CFTR*) gene. The vast majority of deaths and morbidity result from complications of CF-related lung disease(1, 3). Phenotype/genotype studies have demonstrated considerable variability in the severity of lung disease despite patients having the same *CFTR* genotype. This variability may reflect non-*CFTR* genetic modifiers, differences in environmental factors, and access to and the nature of clinical care(18). Genetic modifiers may alter CFTR function, or modify the CF phenotype at the molecular, cellular, systemic or organismal level(19). Studies utilising a combination of candidate gene and genome wide association studies (GWAS) have identified mutations in genes which encode immune mediators (including mannose binding lectin (MBL), transforming growth factor β, interferon-related developmental regulator 1 and interleukin 1), anti-oxidants (e.g. glutathione pathways), ion transporters (e.g. solute carrier family genes), pancreatic β-cell function (transcription factor 7-like 2) and Serpin peptidase inhibitor, clade A (SERPINA)-1 as potentially important modifiers of the CF phenotype(18, 20-24, 323, 324).

In hereditary haemochromatosis (HH), another common clinically important, autosomal recessive condition in Caucasian populations, mutations in the *HFE* gene impact on systemic iron homeostasis by inhibiting the production and release of the regulatory protein hepcidin from the liver in response to iron loading(145). A single point mutation in the *HFE* gene (leading to the C282Y substitution in the HFE protein) accounts for >90% of cases of HH(145). Homozygotes for the C282Y mutation demonstrate the most severe iron loading and phenotype, typified by liver fibrosis and cirrhosis, small joint arthropathy and diabetes mellitus(145). A second *HFE* gene mutation (leading to the H63D substitution), whilst more prevalent in the population, is typically only associated with iron loading when present in combination with the C282Y mutation (i.e. compound heterozygotes), and then only infrequently. The presence of a single *HFE* gene mutation does not usually result in an iron loading phenotype, nevertheless heterozygotes with either a C282Y or H63D mutations may be at increased risk of liver disease, neurodegenerative disease, type II diabetes and malignancy, suggesting either impaired iron handling at a cellular level or some iron-independent function of *HFE*(325-328).

In our earlier small study, subjects with CF who had co-inherited a *HFE* gene mutation had more severe CF lung disease(174). Furthermore, two studies of children with CF have reported an association between the C282Y mutation and the risk of meconium ileus (MI) at birth(173, 175).

This current study further explores the association between *HFE* gene mutations and CF, including CF-related lung disease, risk of CF-related diabetes (CFRD) and bowel obstruction (MI and distal intestinal obstruction syndrome [DIOS]) in adults with CF.

6.3 Methods

Subjects over 18 years of age with CF were recruited from the Adult CF Centre of The Prince Charles Hospital, Brisbane, Queensland, Australia. The study was approved by the Prince Charles Hospital, Human Research and Ethics Committee (HREC/11/QPCH/36) and all subjects provided written informed consent before participating.

Clinical demographics recorded included *CFTR* genotype, age, best lung function (forced expiratory volume in 1 second [FEV_1] and forced vital capacity [FVC]), body mass index (BMI) in the previous 12 months, CFRD, CF-related liver disease (CFLD), history of MI or DIOS and presence of *Pseudomonas aeruginosa* in routine sputum cultures. CFRD was defined broadly to incorporate impaired glucose tolerance, indeterminate glycaemia (raised glucose during an oral glucose tolerance test) and CFRD with or without fasting hyperglycaemia(329). MI was defined as failure to pass meconium in the first days following birth, with associated clinical and radiological features of obstruction(330). DIOS was defined as a history of complete or incomplete intestinal obstruction during the lifetime of the patient, with evidence of a faecal mass at the ileo-caecal junction clinically or radiologically, and associated symptoms of abdominal pain and vomiting(330) (331).

In order to compare lung function predicted values between subjects of variable age, each subject was allocated a lung disease phenotype (mild, severe, neither mild nor severe), based on their age and best FEV_1 predicted value in the year of enrolment, using previously published nomograms(332). In addition, all available historical lung function measurements (from 1998 onwards) for each subject were recorded and used to compare the rate of lung function decline between *HFE* genotype groups.

Venous blood (6 mL) was collected into a lithium heparin tube. Two hundred microlitres of whole blood and 500 µL of plasma were stored at -80^{o}C for later batch analysis of *HFE* genotype and hepcidin level, respectively. Genomic DNA was extracted from 100 µL of stored whole blood using a DNeasy® Blood and Tissue kit (Qiagen) as per the manufacturer's protocol. The presence of C282Y and H63D *HFE* gene mutations was determined by real-time polymerase chain reaction

(TaqMan® primers and Universal PCR Master Mix from Applied Biosystems®) using established methodologies(333). Plasma hepcidin-25 concentration was determined by high-performance liquid chromatography, with online extraction, coupled to tandem mass spectrometry (HPLC-MS) using established methods which have been described in detail previously(334). The assay has an analytical range of 5–100 ng/mL (r^2>0.990) with inter- and intra-day accuracy and imprecision across the analytical range of 95.4–106% and <9.9% respectively. Serum ferritin, soluble transferrin receptor (sTfR), transferrin saturation, total iron and haemoglobin levels were measured from a simultaneously collected blood sample by Queensland Health Pathology Service, Australia. The sTfR to Log_{10}[ferritin] ratio (sTfR ratio) was calculated as marker of iron stores in the setting of systemic inflammation(152).

6.3.1 Statistical Analysis

Data analysis was performed using PASW, Version 18.0 (SPSS Inc. Chicago, IL, USA). Between-group differences of continuous variables were analysed by Mann-Whitney U test. Differences in categorical variables were determined by Chi-squared tests, or by Fishers exact test where the predicted number of subjects in each group was less than five. Differences in lung function decline between the groups were determined by linear, mixed effects modelling. Only subjects in whom a minimum of five lung function measurements over a minimum of two years were available were included in the analysis of rate of lung function decline, in order to limit potential skew from inclusion of subjects with limited data points (median 30 measurements per subject [interquartile range 21-45], median time from first to last measurement 8.8 years [interquartile range 4.5-12.4 years]. To account for the effects of variable *CFTR* genotypes and *P. aeruginosa* infection on outcomes, repeat analysis was performed on sub-groups of subjects who were homozygous for the common F508del *CFTR* gene mutation or infected with *P. aeruginosa*. For the purposes of statistical analysis, patients were segregated into three groups on the basis of their most “severe” single *HFE* gene mutation - normal *HFE* genotype, H63D mutation (H63D heterozygotes and homozygotes), and C282Y mutations (C282Y heterozygotes, homozygotes and compound heterozygotes).

6.4 Results

HFE genotype was determined for 163 adult CF subjects. Sixty-one subjects carried a *HFE* gene mutation (H63D: 33 heterozygotes, 4 homozygotes, C282Y: 20 heterozygotes, 1 homozygote and 3 H63D/C282Y compound heterozygotes) and a normal, wild-type *HFE* genotype was present in 102

patients. The clinical characteristics of subjects are seen in Table 6.1. Longitudinal lung function data were available for 151 subjects.

Table 6-1 Subject demographics

HFE genotype	Wild-type (n=102)	H63D (n=37)	P-value[$]	C282Y (n=24)	P-value[#]
Male gender n (%)	63 (62)	22 (60)	0.81	14 (58)	0.76
Age (years)	27 (23-34)	29 (26-39)	0.09	27 (22-32)	0.58
FEV_1 (Litres)	2.3 (1.8-3.1)	2.4 (1.6-3.1)	0.80	2.0 (1.6-2.5)	0.05
FEV_1 % predicted	66 (48-82)	65 (43-84)	0.80	51 (36-69)	**0.032**
FVC (Litres)	3.8 (3.0-4.7)	3.8 (3.2-4.9)	0.59	3.3 (3.0-3.8)	0.07
FVC % predicted	82 (69-93)	84 (72-95)	0.63	73 (58-81)	**0.023**
BMI	23 (21-25)	23 (21-26)	1.00	21 (19-25)	0.17
CFTR genotype n (%)					
- F508del Homozygote	54 (53)	19 (51)	0.74	13 (53)	0.89
- F508del Heterozygote	39 (38)	13 (35)		9 (38)	
- Other	7 (7)	3 (8)		1 (4)	
- Unknown	2 (2)	2 (5)		1 (4)	
CF Pulmonary Phenotype n (%)					
- Mild	71 (70)	26 (74)	0.75	9 (39)	**0.022**
- Not mild / not severe	25 (25)	8 (23)		11 (48)	
- Severe	6 (6)	1 (3)		3 (13)	
CF-Related Diabetes* n (%)	33 (33)	14 (38)	0.60	14 (58)	**0.022**
CF-Related Liver Disease n (%)	9 (9)	2 (5)	0.49	1 (4)	0.69
CF-Related Pancreatic Insufficiency n (%)	91 (89)	34 (92)	0.76	22 (92)	1.00
Gastrointestinal Complications[^] n (%)	19 (19)	14 (38)	**0.019**	9 (38)	**0.045**
Sputum Microbiology					
- *Pseudomonas aeruginosa*	87 (85)	36 (97)	0.07	22 (92)	0.52

Continuous variables are expressed as median and inter-quartile range. Differences between Wild-type and [$]H63D or [#]C282Y genotypes were examined by Mann-Whitney U-test. Differences between categorical variables were examined by chi-squared or Fishers exact tests, dependent on the number of categories and predicted cell values. *Two subjects with type II diabetes mellitus were excluded from analysis. ^Meconium ileus or distal intestinal obstruction syndrome, *HFE*: Haemochromatosis gene, FEV_1: Forced expiratory volume in one second, FVC: Forced vital capacity, BMI: Body mass index, *CFTR*: Cystic fibrosis transmembrane conductance regulator gene, CF: Cystic fibrosis

6.4.1 Relationship between *HFE* gene mutations and lung function

Subjects with a C282Y mutation had lower FEV_1 (51% versus 66%, p=0.032) and FVC (73% versus 82%, p=0.023) percentage predicted values compared to subjects with a normal *HFE* genotype (Table 6.1). A greater proportion of subjects with a C282Y mutation had a severe lung phenotype compared with subjects who had a normal *HFE* genotype (13% versus 6%, p=0.022). No difference was seen in cross-sectional lung function or pulmonary phenotype between subjects with a H63D and normal *HFE* genotype (Table 6.1).

Subjects with a C282Y HFE mutation experienced an increased rate of decline in FEV_1 and FVC when compared to subjects without a *HFE* gene mutation. Similarly, subjects with a H63D mutation experienced a more rapid decline in FVC than subjects without a *HFE* gene mutation (Table 6.2). When the analysis was limited to F508del *CFTR* homozygotes (n=79), the association with lung function decline (FEV_1 and FVC) in subjects with H63D and C282Y mutations was exaggerated (Table 6.3). Between group differences in rates of lung function persisted in a sub-group analysis limited to subjects infected with *P. aeruginosa* (n=136) (Table 6.4).

Table 6-2 Mixed effect model – Rate of lung function decline

	FEV_1			FVC		
	Estimate	S.E.	P-value	Estimate	S.E.	P-value
Percentages predicted						
Intercept (Wild type)	70.26	2.07		79.87	1.77	
- H63D	74.27	4.05	0.32	84.23	3.46	0.21
- C282Y	61.93	4.88	0.09	72.09	4.16	0.06
Decline per year (Wild type)	-1.71	0.05		-0.79	0.05	
- H63D	-1.80	0.10	0.35	-1.05	0.10	**0.01**
- C282Y	-2.18	0.12	<**0.001**	-1.39	0.12	<**0.001**
Litres						
Intercept (Wild type)	2.80	0.09		3.99	0.11	
- H63D	2.95	0.18	0.40	4.23	0.21	0.26
- C282Y	2.52	0.21	0.18	3.65	0.26	0.18
Decline per year (Wild type)	-0.08	0.002		-0.06	0.003	
- H63D	-0.08	0.004	0.35	-0.07	0.004	**0.002**
- C282Y	-0.11	0.12	<**0.001**	-0.09	0.004	<**0.001**

FEV_1: Forced expiratory volume in one second, FVC: Forced vital capacity, S.E: standard error

Table 6-3 Rate of lung function decline in F508del homozygotes sub-group (n=79).

	FEV_1			FVC		
	Estimate (%)	S.E.	P-value	Estimate (%)	S.E.	P-value
Percentage predicted						
Intercept (Wild type)	72.30	2.86		82.02	2.30	
- H63D	74.49	5.69	0.70	86.48	4.58	0.33
- C282Y	62.75	6.50	0.14	74.19	5.21	0.14
Decline per year (Wild type)	-1.65	0.05		-0.79	0.07	
- H63D	-2.10	0.12	**<0.001**	-1.21	0.14	**0.002**
- C282Y	-2.50	0.15	**<0.001**	-1.80	0.17	**<0.001**
Litres						
Intercept (Wild type)	2.90	0.13		4.10	0.15	
- H63D	3.06	0.26	0.53	4.45	0.30	0.24
- C282Y	2.54	0.30	0.22	3.68	0.35	0.23
Decline per year (Wild type)	-0.08	0.002		-0.06	0.004	
- H63D	-0.10	0.004	**<0.001**	-0.08	0.007	**<0.001**
- C282Y	-0.12	0.006	**<0.001**	-0.11	0.008	**<0.001**

FEV_1: Forced expiratory volume in one second, FVC: Forced vital capacity, S.E: standard error

Table 6-4 Rate of lung function decline in *P. aeruginosa* infected sub-group (n=136).

	FEV_1			FVC		
	Estimate (%)	S.E.	P-value	Estimate (%)	S.E.	P-value
Percentage predicted						
Intercept (Wild type)	69.08	2.25		78.54	1.93	
- H63D	73.49	4.20	0.30	83.58	3.61	0.16
- C282Y	61.95	4.98	0.15	72.10	4.26	0.13
Decline per year (Wild type)	-1.74	0.05		-0.83	0.06	
- H63D	-1.80	0.10	0.53	-1.04	0.11	0.05
- C282Y	-2.19	0.12	**<0.001**	-1.38	0.13	**<0.001**
Litres						
Intercept (Wild type)	2.77	0.10		3.92	0.12	
- H63D	2.92	0.19	0.29	4.18	0.26	0.24
- C282Y	2.52	0.22	0.26	3.64	0.27	0.30
Decline per year (Wild type)	-0.09	0.002		-0.06	0.003	
- H63D	-0.09	0.004	0.40	-0.07	0.005	**0.009**
- C282Y	-0.11	0.005	**<0.001**	-0.09	0.006	**<0.001**

FEV_1: Forced expiratory volume in one second, FVC: Forced vital capacity, S.E: standard error

---

6.4.2 Relationship between *HFE* genotype, CFRD and bowel obstruction.

There was higher prevalence of CFRD in subjects with a C282Y mutation compared to subjects with a normal *HFE* genotype (58% versus 33% respectively, p=0.022). The prevalence of CFRD was not different in subjects with a H63D mutation compared to subjects with a normal *HFE* genotype (Table 6.1). When the analysis was limited to F508del homozygotes, there was no significant association between *HFE* mutations and CFRD (Table 6.5).

A history of MI and/or DIOS was more common in subjects with either H63D or C282Y mutations compared to subjects with a normal *HFE* genotype (38% in each *HFE* mutation group versus 19%, p=0.019 and p=0.045 respectively) (Table 6.1). When the analysis was limited to F508del homozygotes, the increased risk of DIOS persisted in subjects with a C282Y genotype (Table 6.5).

Table 6-5 Subject demographics for F508del homozygous

	Wild Type (n=54)	H63D (n=19)	P-value	C282Y (n=13)	P-value
Male gender n (%)	32 (59)	13 (68)		8 (62)	
Age (years)	27 (22-35)	29 (22-36)	0.49	23 (19-29)	0.12
FEV_1 (Litres)	2.3 (1.9-3.2)	2.5 (1.6-3.8)	0.85	2.0 (1.4-2.7)	0.09
FEV_1 % predicted	69 (69-83)	64 (43-84)	0.69	51 (36-74)	0.08
FVC (Litres)	3.8 (3.0-4.9)	4.2 (3.2-5.2)	0.46	3.3 (2.7-3.7)	0.13
FVC % predicted	82 (71-94)	86 (74-94)	0.63	73 (52-87)	0.07
BMI	22 (21-25)	23 (21-25)	0.95	22 (19-26)	0.49
CF Pulmonary Phenotype n (%)					
- Mild	38 (70)	13 (72)	0.99	4 (31)	**0.018**
- Not mild / not severe	13 (24)	4 (22)		6 (46)	
- Severe	3 (6)	1 (6)		3 (23)	
CF-Related Diabetes n (%)	19 (35)	5 (26)	0.48	6 (46)	0.53
CF-Related Liver Disease n (%)	7 (13)	1 (5)	0.67	1 (8)	1.00
Gastrointestinal Complications^ n (%)	9 (17)	8 (42)	0.05	8 (62)	**0.002**

Continuous variables expressed as median and inter-quartile range. Difference between Wild type and $H63D or #C282Y genotype examined by Mann-Whitney U-test. Differences between categorical variables were examined by chi-squared or Fishers exact tests, dependent on the number of categories and predicted cell values. ^Meconium ileus or distal intestinal obstruction syndrome, *HFE*: Haemochromatosis gene FEV_1: Forced expiratory volume in one second, FVC: Forced vital capacity, BMI: Body mass index, *CFTR*: Cystic fibrosis transmembrane conductance regulator gene, CF: Cystic fibrosis

---

6.4.3 Relationship between *HFE* genotype, systemic iron indices and hepcidin.

Transferrin saturations were significantly higher in subjects with a C282Y mutation compared to those with a normal *HFE* genotype. Otherwise, indices of iron status and hepcidin levels were similar in subjects with different *HFE* genotypes (Table 6.6).

Table 6-6 Biochemical profiles

	Normal Range	*HFE* Genotype				
		Wild Type (n=102)	H63D (n=37)	P-value[$]	C282Y (n=24)	P-value[#]
Haemoglobin (g/L)	135-180	137 (128-145)	138 (132-152)	0.29	131 (120-150)	0.24
WCC (x10^9cells/L)	4-11	8.9 (7.1-11.2)	7.9 (6.0-10.7)	0.25	7.4 (6.2-9.8)	0.07
Neutrophils (x10^9cells/L)	2-8	5.8 (4.1-8.1)	4.7 (3.4-7.5)	0.21	4.6 (3.5-7.1)	0.15
ALP (U/L)	53-128	94 (77-121)	95 (83-113)	0.84	84 (69-121)	0.47
AST (U/L)	<45	22 (18-30)	22 (16-32)	0.65	20 (16-41)	0.75
ALT (U/L)	<35	25 (17-41)	25 (16-48)	0.96	21 (12-50)	0.40
Iron (µmol/L)	12-31	9 (6-14)	11 (7-16)	0.36	11 (8-16)	0.20
Transferrin Saturations (%)	15-45	15 (10-21)	18 (12-25)	0.16	20 (15-27)	**0.011**
Ferritin (µg/L)	30-300	40 (23-67)	43 (28-67)	0.68	53 (39-70)	0.15
STfR (mg/L)	0.8-1.8	1.2 (1.0-1.4)	1.2 (1.0-1.4)	0.52	1.1 (0.9-1.2)	0.05
STfR ratio[#]	<2.0	0.7 (0.6-0.9)	0.7 (0.5-0.9)	0.42	0.7 (0.5-0.8)	0.09
Hepcidin (ng/L)	N/A	7.9 (2.5-24.7)	6.7 (2.9-20.2)	0.53	8.1 (1.8-15.4)	0.47
CRP (mg/L)	<5	4.4 (2.0-12.0)	4.4 (2.2-14.8)	0.40	11.5 (3.8-25.0)	**0.023**

Variables expressed as median and inter-quartile range. Difference between Wild type and [$]H63D or [#]C282Y genotype examined by Mann-Whitney U-test. WCC: White cell count, ALP: Alkaline phosphatase, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, STfR: Soluble transferrin receptor, STfR rato: STFR/Log_{10}[ferritin], CRP: C-reactive protein, N/A: Not available.

6.5 Discussion

In this study, we demonstrate an association between both the C282Y and H63D HFE mutations and an accelerated rate of decline in lung function and occurrence of MI and/or DIOS in subjects with CF when compared to subjects with a normal *HFE* genotype. This disease modulating effect was particularly strong in those patients with the most common F508del homozygote CF genotype. The C282Y mutation, which is usually associated with the greatest degree of iron loading, was associated with a more severe CF clinical phenotype than the H63D mutation, which extended to a weak association with an increased risk of CFRD. These findings support our earlier published work and strongly suggest that the presence of a mutation in the *HFE* gene may modulate CF pulmonary and gastrointestinal disease phenotypes(174).

Identification of gene modifiers in CF may direct new therapeutics and biomarker development(20, 335). A number of modifier genes have been studied in CF with a predominant focus on polymorphisms in genes that are involved in the innate immune system, antioxidant and anti-protease defences, or tissue repair(20). Gene modifiers may contribute to the CF disease phenotype by altering the expression and/or function of CFTR, or they may exert indirect effects. For example, mannose binding lectin (MBL) is an important pathogen pattern recognition receptor in the lung which opsonises invading pathogens to promote immune cell phagocytosis(336). The O/O and XA/O *MBL2* genotypes occur in 10-15% of the CF population and result in significantly reduced MBL production. These two mutations are associated with poorer lung function, an increased risk of *P. aeruginosa* infection, and increased likelihood of death or need for transplantation in CF(337). Several other potential gene modifiers of the CF phenotype have been identified, but these are present in only a small proportion of individuals with CF(18, 20).

The airways of subjects with CF contain increased amounts of iron and the dominant CF pulmonary pathogen, *P. aeruginosa*, has an obligatory requirement for iron for replication and respiration(169, 179, 196, 265, 338). *In vitro* studies demonstrate that CF bronchial epithelial cells "leak" iron at their apical surface, which in turn promotes *P. aeruginosa* biofilm growth(180). The potential importance of iron homeostasis in CF supports the study of the role of *HFE* gene mutations, especially given the central importance of HFE in controlling body iron levels. Furthermore, in contrast to many other previously explored modifying factors, iron homeostasis may be modifiable with therapeutic manipulation through dietary modification or potentially, the use of agents that chelate iron(338, 339).

Chronic *P. aeruginosa* infection is strongly associated with accelerated rate of lung function decline in CF and represents a potential confounder in modifier gene studies(94). The prevalence of *P. aeruginosa* infection in our study was similar among subjects with each *HFE* genotype (Table 6.1) and when analysis was limited to subjects infected with *P. aeruginosa*, rates of decline in lung function remained highest in subjects with a C282Y mutation (Table 6.4).

The risk of development of bowel obstruction (MI or DIOS) has been ascribed to modifier genes in CF previously, and importantly gastrointestinal complications in childhood have been shown to predict for worse long term pulmonary outcomes(20, 324, 340). A relationship between an increased risk of MI in infants and the C282Y mutation was originally suggested in an Irish study(173, 175). However, this association was not supported by a more recent multi-national GWAS, which identified five SNPs (three on chromosome 1 and two on the X chromosome), in the

region of two solute carrier family genes (*SLC6A14* and *SLC26A9*) that were associated with an increased risk of MI, but did not identify any SNPs in the region of the *HFE* gene(324). The GWAS included more subjects (> 3000 individuals) than our current study and limited the analysis to occurrence of MI in newborns, whereas our study has broadened the clinical diagnosis to include bowel obstruction secondary to DIOS. (341). A further analysis of the GWAS data is warranted to include patients with a history of DIOS and MI, but the current findings are in agreement with our earlier study conducted in the CF population living in Tasmania, and our studies are also supported by earlier published work from Ireland that showed an increased risk of MI in patients with a C282Y *HFE* mutation, which we now demonstrate also extends to patients with the H63D *HFE* genotype.

We observed an association between the C282Y mutation and prevalence of CFRD, supporting the findings of our earlier work. In the general population, the development of type II diabetes is influenced by genetic and environmental factors(342). Single nucleotide polymorphisms (SNPs) in several genes (including *SLC26A9* and *TCF7L2*) have been linked to an increased risk of developing type II diabetes and these SNPs also appear to increase the risk of developing CFRD(20). The C282Y mutation and iron loading independent of *HFE* genotype have been identified as additional risk factors for the development of type II diabetes and *HFE* mutations are associated with increased diabetes-related morbidity and mortality in the general population(343-345). Additional investigation of the relationship between *HFE* gene mutations and CFRD is required in a larger CF population to further define this association.

Only four subjects in the current study had a *HFE* genotype typically associated with clinically significant systemic iron loading (one C282Y/C282Y homozygote and three C282Y/H63D compound heterozygotes), nevertheless, there is precedence for single *HFE* gene mutations impacting disease phenotype. For example, subjects with hepatitis C virus (HCV) infection who are heterozygous for the C282Y mutations display increased serum ferritin, liver iron levels and hepatic fibrosis compared with HCV infected subjects with a normal *HFE* genotype(327). An association with increased liver disease severity is also evident in *HFE* mutation heterozygotes who have co-existent porphyria cutanea tarda and non-alcoholic steatohepatitis(346, 347). Abnormal cerebral iron homeostasis is evident in the pathogenic lesions associated with many neurodegenerative diseases and consistent with the importance of iron to central nervous system disease, heterozygosity for the H63D mutation is associated with an increased risk of motor neurone disease(328), (348).

In our cohort, serum hepcidin levels and markers of iron status were not significantly different in CF subjects with different *HFE* mutations, suggesting that subjects with predisposing *HFE* mutations were not iron loaded systemically at the time the study was performed. Nevertheless, it remains possible that a single *HFE* mutation, although not sufficient to disturb iron concentrations at the systemic level in CF, may promote disease by further exacerbating iron mishandling caused by *CFTR* mutations at the cellular level. Our data suggest a greater influence for the C282Y, compared to H63D mutation on CF disease phenotype, and the overall impact of *HFE* mutations appeared to be greatest in the gastro-intestinal tract, followed by respiratory and endocrine systems. The explanation for this apparent difference in organ susceptibility to dysregulated iron homeostasis is uncertain and requires further investigation.

The study has a number of limitations. Firstly, this was a single centre study and included a relatively small population compared to other large, multi-centre gene modifier studies which have recruited up to 2500 patients in order to maximise statistical power and limit the potential for Type I errors(21, 349). We have, however, replicated our earlier findings generated in a separate CF population of an association between C282Y and H63D mutations and disease severity, and the link between iron and disease severity has strong biological rationale. Furthermore, large, multicentre studies may be negatively impacted by the confounding effect of different environmental and treatment exposures between centres(349). The second limitation of our study is that recruitment was not limited to CF subjects with only the most common F508del homozygote *CFTR* genotype. However, when a sub-group analysis was undertaken in F508del homozygotes, the presence of a *HFE* mutation had an exaggerated effect on the rate of decline of lung function (Table 6.3), while the trend in other parameters remained similar to the larger cohort (Table 6.5 and 6.7). Thirdly, to maximise recruitment in the current study, blood testing was not always performed during clinical stability. As a consequence, subjects with a C282Y mutation were predominantly recruited during treatment of a pulmonary exacerbation, reflecting the fact that their health was generally poor at the time of study. The associated acute phase response may have perturbed a number of iron indices and reduced the ability to detect differences between groups by reducing systemic iron levels in the C282Y group as part of the innate immune iron withholding response. Nevertheless, the sTfR ratio is considered to be an accurate surrogate marker of body iron stores in the presence of inflammation, and values for this parameter were not different between groups(149).

Table 6-7 Biochemical profiles of DF508del homozygous subjects

	Normal Range	HFE Genotype				
		Wild Type (n=52)	H63D (n=19)	P-value[$]	C282Y (n=13)	P-value[#]
Haemoglobin (g/L)	135-180	137 (130-144)	138 (135-157)	0.16	130 (124-149)	0.40
WCC (x10^9/L)	4-11	9.3 (7.0-11.2)	7.8 (5.3-10.1)	0.29	7.4 (5.7-9.0)	0.06
Neutrophils (x109/L)	2-8	6.0 (4.1-8.3)	4.7 (3.0-7.3)	0.19	4.4 (3.6-5.6)	0.06
ALP (U/L)	53-128	97 (79-126)	96 (90-119)	0.77	85 (65-109)	0.17
AST (U/L)	<45	24 (18-30)	21 (16-34)	0.68	19 (16-38)	0.58
ALT (U/L)	<35	26 (19-42)	24 (16-44)	0.55	18 (11-52)	0.23
Iron (µmol/L)	12-31	9 (6-14)	10 (8-15)	0.28	12 (8-18)	0.20
Transferrin Saturations (%)	15-45	15 (10-21)	18 (12-23)	0.21	23 (16-31)	**0.038**
Ferritin (µg/L)	30-300	44 (23-70)	45 (25-72)	0.88	55 (40-68)	0.61
STfR (mg/L)	0.8-1.8	1.2 (1.0-1.4)	1.2 (1.0-1.3)	0.70	1.1 (0.9-1.2)	0.26
STfR ratio[#]	<2.0	0.7 (0.6-0.9)	0.7 (0.5-0.9)	0.72	0.7 (0.5-0.9)	0.52
Hepcidin	N/A	10.1 (3.4-28.0)	8.2 (3.1-28.7)	0.88	4.3 (1.6-15.1)	0.10
CRP (mg/L)	<5	5 (2.0-10.0)	4.4 (2.0-11.0)	0.98	14 (4.2-26.0)	0.08

Variables expressed as median and inter-quartile range. Difference between Wild type and [$]H63D or [#]C282Y genotype examined by Mann-Whitney U-test. WCC: White cell count, ALP: Alkaline phosphatase, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, STfR: Soluble transferrin receptor, STfR rato: STFR/Log_{10}[ferritin], CRP: C-reactive protein

---

6.6 Conclusion

In summary, CF subjects who carry a *HFE* gene mutation, and in particular the C282Y substitution, demonstrate an accelerated decline of lung function and increased prevalence of MI and/or DIOS. Our findings support the need for mechanistic studies to further determine the effect of *HFE* mutations on iron homeostasis in CF. They also suggest that screening the CF population for the presence of *HFE* mutations may be useful to identify patients who may be at particular risk of iron-related disease modulation. Further research is needed to determine whether interventions that limit iron such as dietary modification, including avoidance of iron supplements, or iron chelation therapy should be introduced to potentially ameliorate *HFE* mutation-mediated modulation of CF disease outcomes.

Chapter 7. Discussion

7.1 Introduction.

The work presented in this thesis had three overarching aims; i) Determine the relationship between treatment practices in Australian CF centres and rates of *P. aeruginosa* antibiotic resistance, ii) Determine bacterial population and host immune related factors which may influence pulmonary infection and response to anti-microbial therapy, and iii) Determine the relationship between systemic and pulmonary iron homeostasis and CF lung disease severity. The overall aim was to investigate antibiotic prescribing practices, lung microbiome dynamics and host immune factors that may contribute to chronic infection with *P. aeruginosa* in CF.

To address **Aim One**; in chapter 2, data from the Australian clonal *P. aeruginosa* in CF study (ACPinCF) and the Australian CF Data Registry (ACFDR) were utilised to determine treatment related factors which may promote antibiotic resistance in children and adults infected with *P. aeruginosa*.

To address **Aim Two**; in chapter 3, metagenomic techniques were applied to determine the bacterial composition of CF sputum samples. The airway microbiome composition was correlated with clinical demographics, and the changes in composition of the microbiome in response to intravenous antibiotics was examined by performing serial measures in subjects undergoing antibiotic treatment for a pulmonary exacerbation. In chapter 4, multi-colour flow cytometry was used to phenotype peripheral blood lymphocytes from CF subjects and healthy controls. Differences in lymphocyte sub-sets were compared between groups, and in subjects with CF, lymphocyte sub-sets were correlated with parameters of infection and increased disease severity. Finally in chapter 5, the presence of bio-active metals in the sputum of subjects with CF and NCFB was determined by ICP-MS and compared to concentrations found in induced sputum samples from healthy controls. Metal concentrations were correlated with parameters of increased airway inflammation and tissue damage.

Finally, to address **Aim Three**; in chapter 5, the concentration of iron in the CF airway was correlated with clinical demographics and airway infection, inflammation and tissue injury. In chapter 6, the role of the *HFE* gene as a potential modifier of CF disease phenotype was explored.

7.2 Key Findings of this Thesis.

7.2.1 Trends in *P. aeruginosa* antibiotic resistance in Australian CF centres.

7.2.1.1 Key Findings

The findings presented in Chapter 2, demonstrate the use of antibiotic therapies to manage CF airways disease in patients infected with *P. aeruginosa* vary widely between different paediatric and adult CF centres across Australia. Within paediatric centres the rates of MARPA correlated with the use of intravenous antibiotics. In both adult and paediatric centres, the treatment centre in which treatment was delivered was an independent predictor of intravenous antibiotic usage and of MARPA.

Adult and children infected with a one of the major Australian shared strains (AUST-01 or AUST-02) had an increased treatment burden. Furthermore patients infected with AUST-01 and AUST-02 were prescribed more maintenance therapies and intravenous antibiotics, and were more likely to possess a MARPA isolate.

7.2.1.2 Limitations

Due to the complexity of the ACPinCF study it was not feasible to perform all the antibiotic sensitivity testing in a single laboratory, nor was it possible to test the antibiotic sensitivity of individual strains which were genotyped. Consequently, differences in testing techniques employed between states may have accounted for some of the differences in antibiotic resistance profiles. Similarly, the antibiotic resistance profile generated by the local laboratory may not have been reflective of the antibiotic resistance of the genotyped strains.

This study was cross-sectional and the chronicity of infection in each subject was not determined, consequently, infection may have been transient in some subjects. This is important as subjects with transient infection are less likely to be infected with a resistant strain, and more likely have mild disease and respond to eradication regimens(116, 197, 198).

Information about which intravenous antibiotics were prescribed, or the specific reason for administration was not available. This additional information would been advantageous in determining the impact of particular antibiotic on induction of resistance. Equally, in some centres,

intravenous antibiotic administration may have been performed regularly (e.g. every 3 months) and not driven by clinical indications (e.g. treatment limited to the management of a pulmonary exacerbation). This is important, as different treatment approaches may account for the variable rates of intravenous antibiotic usage between sites and may potentially impact antibiotic resistance(350).

Finally, maintenance therapies were recorded from hospital dispensing data systems and patient medical records, which may not accurately reflect treatment adherence and thus the actual intensity of exposure to antibiotics.

7.2.1.3 Implications for Future Work

Worldwide, quality improvement projects have been undertaken in which patient care has been benchmarked against high performing centres(203-205). These projects have proven valuable in identifying key metrics of "best practice" which can be used to drive improvements in service delivery(203). Furthermore, in the US and Europe consensus recommendations for managing pulmonary exacerbations and prescribing maintenance therapies have been published to promote consistent and evidence-based practice(127, 202, 245). However, similar guidelines are not currently in place in Australia.

The findings contained in chapter 2 are important, as they highlight apparent, wide variations in treatment practices between different Australian CF centres. Furthermore, the results indicate (especially among paediatric centres) that treatment practices may influence the development of MARPA. Although the clinical significance of MARPA remains a matter of debate(238, 239), increased antibiotic usage at an early age may impact on long term treatment related morbidity, including the development of antibiotic allergy and toxicity(5, 6, 351). Despite the variable treatment approaches employed, there were few differences in health related outcomes between centres, however, longitudinal studies will be important in determining whether the early benefits of aggressive antibiotic treatment in early life are sustained into adulthood. Nevertheless, the results of the study, represent an opportunity to open dialogue between CF treatment centres within Australia, to consider the development of national consensus treatment guidelines to maximise clinical benefit whilst minimising development of multi-antibiotic resistance.

Antibiotic sensitivity testing methodologies employed, vary between the different microbiology laboratories involved in the routine testing of CF sputum samples, which may impact on antibiotic

resistance profiles generated for a *P. aeruginosa* strain. The adoption of standardised national susceptibility testing would allow the resistance profiles generated at different sites to be directly compared. At the population level, this would be advantageous in monitoring resistance trends and assist in the identification of 'outbreaks' of resistant strains. For the individual with CF, their personal antibiotic resistance profile should perhaps be monitored over time to allow the early detection of antibiotic resistance development and consideration of a change in antibiotic usage. Although this study focused in particular on *P. aeruginosa* infection, standardisation of susceptibility testing may be of equal importance for the monitoring of other CF airway pathogens.

Finally, the two major Australian shared strains were associated with increased treatment burden. Worldwide, a number of shared strains identified in other populations have been shown to be associated with increased morbidity and these observations have resulted in patient segregation based on strain type(223). Limited data are currently available about the clinical impact of the major Australian shared strains(352), and longitudinal monitoring of patients infected with these strains will be of critical importance in informing the development of infection control policies, which in turn has substantial implications for resources such as physical space (i.e. single in-patient rooms for all CF patients) in CF centres.

7.2.1.4 Proposed Future Work

i) Prospective, longitudinal follow-up of paediatric subjects, to determine if early, aggressive treatment of *P. aeruginosa* is associated with better outcomes, despite the development of MARPA.

ii) Prospective, longitudinal follow-up of subjects infected with a shared *P. aeruginosa* strain, to determine whether these strains are associated with worse long-term outcomes.

iii) Develop national consensus guidelines for antibiotic susceptibility testing in CF, to allow direct comparison of resistance profiles between different regions and for the same patient tested at different treatment centres.

iv) Develop national consensus "best practice" guidelines for the management of chronic *P. aeruginosa* infection and treatment of pulmonary exacerbations.

7.2.2 Response of the CF lung Microbiome to intravenous antibiotics.

7.2.2.1 Key Findings

The findings presented in Chapter 3, demonstrate in a group of adult CF subjects infected with *P. aeruginosa* on standard microbiological cultures, *Pseudomonas spp.* dominated the lung microbiome determined by molecular analysis in 94% of cases. Anaerobic species were also commonly present in the sputum, with *Prevotella* and *Veillonella spp.* being present in 72% and 78% of samples prior to antibiotic treatment respectively. However, the relative abundance of anaerobic species was generally low. Sputum samples with high relative abundance of *Pseudomonas spp.* tended to have low overall microbial diversity. Conversely, increase relative abundance of *Streptococcus spp.* and *Prevotella spp.* correlated with increased microbial diversity.

In subjects experiencing a pulmonary exacerbation, following three days of intravenous antibiotic therapy, the relative abundance of *Pseudomonas spp.* in sputa was reduced and a reciprocal increase in microbial diversity was seen. However, despite ongoing antibiotic pressure, by the end of the first week of treatment, the relative abundance of *Pseudomonas spp.* and overall microbial diversity returned to pre-treatment levels.

Overall, the findings of this study suggest that intravenous antibiotics for the treatment of a pulmonary exacerbation have only a short-lived effect on the lung microbiome. In particular, there is an apparent recrudescence of *Pseudomonas* infection despite ongoing antibiotic pressure. These results add significantly to previous published work in this field. The findings challenge the convention of treating subjects with CF undergoing a pulmonary infection with a two week course of the same intravenous antibiotics and suggest alternate strategies such as rapidly cycling different classes of antibiotics should be explored.

7.2.2.2 Limitations

The results presented in this chapter are largely based on the relative abundance of each bacterial species, expressed as a percentage of the whole microbiome and do not reflect the absolute number of bacteria present. Consequently, confident comment cannot be made on whether changes in bacterial density follows a similar pattern as the relative abundance. However, in a sub-group of recruited subjects, there was a significant positive correlation between the relative abundance of

Pseudomonas spp and quantitative *P. aeruginosa* bacterial load (r^2 = 0.24, p=0.04), which supports the conjecture that an increase in relative abundance of *P. aeruginosa* is accompanied by an increase in actual bacterial numbers.

Secondly, the methods employed to sample and process sputum samples for inclusion in metagenomic studies are currently subject to optimisation. A matter of particular debate is the use of PMA to cross-link (and exclude) eDNA from biological samples(272, 273). There exists, a theoretical concern the extracellular DNA released from non-viable bacteria may skew the results of microbiome studies, especially when the antibiotic bacterial killing effects are studied(273). In the study included in this thesis, in order to minimise the possible effects of extended post-collection processing on the bacterial content of samples, we elected not to perform PMA processing. This decision did not limit our ability to detect differences in the lung microbiome in response to antibiotics. However, in the future, it will be important for researchers in this field to agree upon standardised testing methodologies to allow direct comparisons of results between studies(353).

Finally, this study was performed in adult subjects, with moderately severe lung disease, infected with *P. aeruginosa*, and the findings may not be applicable in younger subjects with milder lung disease, or in subjects who do not have a *P. aeruginosa* infection. Further studies that examine the lung microbiome and how it responds to intravenous antibiotics in subjects who do not culture *P. aeruginosa* are required.

7.2.2.3 Implications for Future Work

Conventionally, CF subjects experiencing an infective pulmonary exacerbation are treated with a two week course of intravenous antibiotics(127). Typically, subjects experience progressive symptomatic improvement and improved lung function over the course of treatment. However, the results presented in this chapter suggest that the lung microbiome may rapidly become tolerant to intravenous antibiotics and challenges the convention of prescribing a prolonged course of the same intravenous antibiotics to all patients being treated for a pulmonary exacerbation. The results of this study may support the prescription of shorter courses of antibiotics, or the adoption of alternate antibiotic prescription strategies e.g. changing the antibiotics prescribed during the treatment of a pulmonary exacerbation. However, such treatment strategies will require careful investigation, in particular for the occurrence of adverse effects, including the potential for increased rates of antibiotic allergy and toxicity, and development of MARPA(5, 6, 235, 351). Furthermore, if clinical improvements cannot be attributed to changes in the lung microbiome, the clinical beneficial effects

of adjuvant therapies administered alongside antibiotics, including airway clearance techniques, use of mucolytics, rehydration and nutritional supplementation, merit greater consideration in future studies. Finally, as clinical improvements do not correlate with changes in airway microbiome, it may be that the absolute number of each bacteria species in the airway is less important than other factors, such as the virulence of the components of the microbiome and the effect that this exerts upon immune response and airway inflammation(269).

7.2.2.4 Proposed Future Work.

i) Perform similar lung microbiome studies in subjects with milder airways disease and also in subjects before the establishment of chronic *P. aeruginosa* infection.

ii) Examine the effects of non-antimicrobial adjunctive therapies on the airway microbiome and pulmonary inflammation.

iii) Randomised controlled trials, which compare the effects of conventional and alternate antibiotic strategies (e.g. rapidly cycling antibiotics) on the airway microbiome.

iv) Studies to explore the effects of antibiotics on the "virulence" of the component of the airway microbiome.

7.2.3 Lymphocyte Phenotype in CF

7.2.3.1 Key Findings

The findings presented in Chapter 4, demonstrate that in comparison to healthy controls, the blood lymphocyte population in subjects with CF contains a lower percentage of MAIT cells and a higher percentage of γ/δ T-cells. In CF subjects, a lower percentage of MAIT cells was associated with *P. aeruginosa* infection, increased lung disease severity and acute pulmonary exacerbations. The absolute numbers of γ/δ T-cells (but not proportion of lymphocytes that were γ/δ T-cells) also correlated with worse lung function.

Total lymphocyte counts were lower in CF subjects undergoing treatment for a pulmonary exacerbation, when compared to stable subjects, and in CF a reduced lymphocyte count correlated with worse lung function.

Overall the results of this chapter provide the basis for future investigations into the potential utility of MAIT cells as a biomarker in CF, and more detailed mechanistic studies to determine whether abnormal MAIT cell responses are implicated in the impaired pulmonary response to infection in CF that allows chronic infection to become established.

7.2.3.2 Limitations

The study presented in chapter 4 was cross-sectional, consequently, it is not possible to determine from the results, whether a causal relationship exists between reduced MAIT cell numbers, chronic bacterial infection and disease severity. Nevertheless, previous authors have determined that MAIT cells provide an important, early response to infection, prior to a mature, adaptive immune responses, which strongly suggests MAIT cells are worthy of further investigation(71, 72).

The study included only a limited number of subjects in the sub-group analysis of stable disease compared with patients undergoing treatment for a pulmonary exacerbation and only five subjects not infected with *P. aeruginosa*. Consequently, type I statistical errors are possible in the analysis of these outcomes. Furthermore, all the CF subjects included in the current study were adults, with chronic airway infection. As a result, it was not possible to determine whether the reduction of MAIT cell number was as a consequence of infection, or whether a primary defect in innate T-cells numbers may be present in subjects with CF, which thus predisposes to bacterial infection. Future studies, which include young patients will be important in identifying whether defects in innate T-cell immunity precede infection.

As circulating lymphocyte populations may not accurately reflect airway populations it is possible that reduced numbers occur as a result of increased tissue recruitment to sites of infection(276). Studies which correlate airway biopsy and circulating lymphocyte numbers will be of great importance in elucidating the role of lymphocytes in disease pathogenesis.

Finally, this study was primarily designed to be ‘hypothesis generating’ and did not examine measures of lymphocyte function and in particular, did not investigate the proportion of the lymphocytes specifically primed to respond to CF airway pathogens.

7.2.3.3 Implications for Future Work

Despite the preliminary nature of the findings of this study, the results are of great potential interest, and provide several exciting avenues for future research into lymphocyte responses in CF.

The different concentrations of MAIT cells between healthy and CF subjects, and the correlation of MAIT cell proportions with lung disease severity and clinical stability in CF, suggest that MAIT cell percentage may be useful as a biomarker of pulmonary infection in CF. In this setting, a strength of monitoring MAIT cells numbers is the restricted responsiveness of these cells to bacterial antigens(69), which for instance could be utilised to differentiate between pulmonary exacerbations triggered by viral or bacterial stimuli. The research presented in this chapter was limited to CF, however, differentiating between viral and bacterial triggered exacerbations is important in other disease setting (e.g. asthma, chronic obstructive pulmonary disease) and future research should explore MAIT cell proportions in other pulmonary diseases.

Reduced proportions of MAIT cells in the blood of CF patients may occur as a result of reduced lineage differentiation, increased peripheral destruction or preferential migration from the blood to tissue. Determining the mechanism by which MAIT cell numbers are reduced may inform the development of therapeutic strategies based on augmenting MAIT cell numbers or function.

Finally, blood MAIT cell concentration may not reflect pulmonary (airway) concentration and studies which determine the presence of MAIT cells in airway biopsy are required to determine whether these cells are important in protecting the lung from infection, although such studies are limited by ethical considerations in CF.

7.2.3.4 Proposed Future Work

i) *Ex vivo* studies of MAIT cells from subjects with CF to determine whether these cells are primed to respond to CF pathogens, with a focus on their response to *P. aeruginosa*.

ii) *Ex vivo* studies to compare the adaptive responses of MAIT cells isolated from the blood of CF and healthy control subjects in response to stimulation by *P. aeruginosa* (and other CF pathogens).

iii) Study MAIT cell proportions in children and subjects with mild lung disease to determine whether differences exist prior to the onset of chronic infection.

iv) Longitudinal study to determine the stability of MAIT cell proportions in the blood of CF subjects over time, and variability in MAIT cell number between periods of stable disease and pulmonary exacerbations.

v) Examination blood MAIT cells proportion in other respiratory disease settings, with a particular focus on determining their utility for differentiation between viral and bacterial exacerbations.

7.2.4 Bioactive metals in the CF airways.

7.2.4.1 Key Findings

The findings presented in chapter 5 demonstrate that airway secretions from subjects with CF and NCFB contain increased concentrations of iron, zinc, magnesium and calcium, when compared to healthy control subjects. Furthermore in subjects with CF and severe airways disease, increased concentrations of molybdenum were found.

Sputum concentrations of magnesium, iron, zinc, calcium and copper correlated with increased concentrations LDH, which was measured as a surrogate markers of local tissue damage(308). Furthermore magnesium, iron and zinc concentrations correlated with increased concentrations of the pro-inflammatory cytokine IL-8. Airway biometal concentrations were not related to the concentration of atmospheric particulate matter, and were present in substantially higher concentrations than previously reported to be present in serum(303-305). Taken together these results suggest that biometals may arise from local tissue destruction and/or the breakdown of inflammatory cells within the airways.

Finally, it was noteworthy that in two subjects who received intravenous iron infusions for the treatment of severe iron deficiency anaemia, sputum iron concentrations were substantially higher in samples collected after the infusion, when compared to initial samples.

7.2.4.2 Limitations

Results presented in this chapter were based on expectorated sputum samples (and induced sputum in health controls) as this is the form of sampling most readily accessible in clinical practice. However, sputum sampling is intrinsically subject to sampling variability and may be confounded by oropharyngeal contamination. However, established methodology was applied to sample collection, in which sputum plugs were carefully separated from saliva, and this approach has been shown to minimise salivary contamination(322).

CF and NCFB subjects included in this study were adults with severe lung disease, which may have limited the ability to detect differences in sputum metal concentrations between stable state and during pulmonary exacerbations.

Finally, it is not possible to determine from the results of this study whether the metal concentrations in the airways are a results of airway inflammation, or whether the presence of bio-active metals in the airways precedes and contributes towards tissue damage by stimulating the generation of reactive oxygen species.

7.2.4.3 Implications for Future Work

The results in this chapter confirm the results of previous authors in finding increased concentrations of zinc and iron in the airways of subjects with CF(176-179, 216). Determination of the concentration of bio-active metals in the airways has two implications of major importance for future research. Firstly, it furthers our understanding of the microenvironment of the lung, which will inform on the development of more accurate culture models for the study of bacterial behaviour *in vitro*. It is hoped that developing accurate *in vitro* models will assist in more accurately predicting phenotypic adaptation of pathogenic bacteria and their response to antimicrobials *in vivo*. Secondly, a number of biometals are essential to the survival of bacteria. Identifying metals which are associated with severe disease, may suggest opportunities for the development of novel therapeutic agents based on interference with bacterial access to specific metals(196).

The identification of high concentrations of molybdenum in the sputum of CF subjects with severe disease is potentially an important finding. In bacteria, molybdenum is a co-factor of nitric reductase, which is an essential enzyme in anaerobic respiratory pathways(298). Molybdenum was not detected in the airways of healthy controls, or CF subjects with milder airways disease, which

may suggest that it is non-essential for normal airway physiology. Consequently, limiting the availability of molybdenum to bacteria, may represent a target for the development of novel therapeutic agents against anaerobic pulmonary infections.

7.2.4.4 Proposed Future Work.

i) Develop *in vitro* culture models with media containing bio-active metal concentrations which reflect *in vivo* airway concentrations.

ii) Explore the expression of molybdenum co-factors and nitric reductase in aerobic and anaerobic *P. aeruginosa* cultures, grown in media containing molybdenum and in molybdenum depleted medium.

iii) Study the therapeutic potential of high affinity chelators targeting specific metals *in vitro* and in animal infection models.

7.2.5 *HFE* as a Gene Modifier in CF

7.2.5.1 Key Findings

In chapter 6 it is demonstrated that CF subjects who carry a *HFE* gene mutation experience an accelerated rate of lung function decline and are at increased risk of DIOS. Furthermore, rates of CFRD were higher in subjects with a C282Y mutation compared to healthy controls.

Peripheral blood, iron and hepcidin concentrations were not different between subjects with difference *HF*E genotypes. This suggests that if *HFE* gene mutations modify the CF phenotype, the mechanism of action may be distinct from its effect on systemic iron stores, perhaps by impacting on iron homeostasis at a cellular level.

The results presented in this chapter are consistent with the results of earlier, smaller, studies in this field and supports the need for further research into how *HFE* gene mutations may potentially modify the CF phenotype(173-175).

7.2.5.2 Limitations.

This is the largest study to date to examine the effects of *HFE* genes on the CF phenotype, however, this only included 51 subjects who carried a *HFE* mutation (only 3 had a potential HH disease causing genotype) and the results may be subject to type I errors. Nevertheless, the study was performed in a single centre, thereby limiting the confounding effects of different environmental and treatment approaches on the results. In addition, the results are consistent with those of previous studies in the field(173-175).

To improve the power of the study, recruitment was not limited to subjects with the same *CFTR* mutation and the uneven distribution of mild CF genotypes may have influenced the outcome of the study. However, *CFTR* genotypes were evenly distributed between subjects with different *HFE* genotypes and sub-group analysis limited to DF508del homozygotes demonstrated trends consistent with the whole group data.

Finally, only the two most common *HFE* gene mutations were screened for, and it is possible some subjects carried another HH causing gene mutation. However, these mutations are rare and generally associated with mild HH phenotypes, and even if present are unlikely to have had a substantial impact on the results(354, 355).

7.2.5.3 Implications for Future Work

Studying the influence of modifier genes on the phenotype of a genetic disorder is important to improve our understanding of the pathophysiology of disease and to identify potential new therapeutic strategies.

In earlier studies, as well as in Chapter 5 of the current thesis, increased concentrations of iron have been identified in the CF airways(176-179, 356). Furthermore, access to iron has been shown to have a powerful influence upon the phenotypic adaptation of *P. aeruginosa*(179, 265, 338).

The results presented suggest the *HFE* gene mutations identified, did not influence systemic iron stores. However, *HFE* mutations, may exert more subtle effects on iron homeostasis at the cellular level, which influence iron handling and immune responses, and increase the risk of infection(221,

357, 358). Consequently, future laboratory based studies could focus on determining the effects of *HFE* mutations at the cellular level in CFTR mutant cells

Finally, high rates of iron deficiency have been reported in subjects with CF and prescription of iron replacement therapy is common practice(169). There is currently limited information on the effects of iron replacement on airway iron availability and the work that has been performed has not considered the possible influence of *HFE* mutations(167, 172). Future clinical studies, should examine the relationship between *HFE* gene mutations and the availability of iron in the CF airway and determine whether iron supplementation regimens should be modified to prevent possible adverse-effects, associated with iron replacement in this potentially 'at risk' population.

7.2.5.4 Proposed Future Work.

i) Develop mouse models which combine *HFE* and *CFTR* mutations in order to study the effects of the combined mutations on systemic and cellular iron handling, infection and gastrointestinal complications. These studies are underway at QIMR-Berghofer and have received NHMRC funding in 2014.

ii) Determine whether *HFE* mutations correlated with increased sputum iron concentrations, airway inflammation and infection in subjects with CF.

iii) Prospective study to screen CF subjects for *HFE* mutations prior to the commencement of iron supplementation, in order to develop strategies to avoid over-zealous iron replacement in subjects susceptible to adverse effects from systemic iron loading.

7.3 Concluding Remarks

The work presented in this thesis provides new insights into therapeutic, bacterial and immune factors, which may promote the persistence of *P. aeruginosa* infection at the population, individual patient and cellular level in CF. The findings presented identify a number of important avenues for future research, which may result in the development of improved techniques for monitoring (biomarkers) and treatment (targeting iron and other biometals) of CF related lung disease.

References.

1. Cystic Fibrosis Foundation patient Registry (2010): Annual Data Report. Bethesda, Maryland, USA. 2012.
2. O'Sullivan BP, Freedman SD. Cystic fibrosis. Lancet. 2009;373(9678):1891-904.
3. Cystic Fibrosis in Australia 2012: 15th Annual report Australian Cystic Fibrosis Data Registry. Baulkham Hills, NSW, Australia2013.
4. Merlo CA, Boyle MP, Diener-West M, Marshall BC, Goss CH, Lechtzin N. Incidence and risk factors for multiple antibiotic-resistant *Pseudomonas aeruginosa* in cystic fibrosis. Chest. 2007;132(2):562-8.
5. Parmar JS, Nasser S. Antibiotic allergy in cystic fibrosis. Thorax. 2005;60(6):517-20.
6. Prayle A, Watson A, Fortnum H, Smyth A. Side effects of aminoglycosides on the kidney, ear and balance in cystic fibrosis. Thorax. 2010;65(7):654-8.
7. Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, Grzelczak Z, et al. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science. 1989;245(4922):1066-73.
8. Kim Chiaw P, Eckford PD, Bear CE. Insights into the mechanisms underlying CFTR channel activity, the molecular basis for cystic fibrosis and strategies for therapy. Essays in biochemistry. 2011;50(1):233-48.
9. Berdiev BK, Cormet-Boyaka E, Tousson A, Qadri YJ, Oosterveld-Hut HM, Hong JS, et al. Molecular proximity of cystic fibrosis transmembrane conductance regulator and epithelial sodium channel assessed by fluorescence resonance energy transfer. The Journal of biological chemistry. 2007;282(50):36481-8.
10. Gentzsch M, Dang H, Dang Y, Garcia-Caballero A, Suchindran H, Boucher RC, et al. The cystic fibrosis transmembrane conductance regulator impedes proteolytic stimulation of the epithelial Na+ channel. The Journal of biological chemistry. 2010;285(42):32227-32.
11. Reddy MM, Quinton PM. Functional interaction of CFTR and ENaC in sweat glands. Pflugers Archiv : European journal of physiology. 2003;445(4):499-503.
12. Rogan MP, Stoltz DA, Hornick DB. Cystic fibrosis transmembrane conductance regulator intracellular processing, trafficking, and opportunities for mutation-specific treatment. Chest. 2011;139(6):1480-90.
13. CFMD. Cystic Fibrosis Mutation Database 2012. Available from: http://www.genet.sickkids.on.ca/StatisticsPage.html.

14. McKone EF, Emerson SS, Edwards KL, Aitken ML. Effect of genotype on phenotype and mortality in cystic fibrosis: a retrospective cohort study. Lancet. 2003;361(9370):1671-6.
15. Derichs N. Targeting a genetic defect: cystic fibrosis transmembrane conductance regulator modulators in cystic fibrosis. European respiratory review : an official journal of the European Respiratory Society. 2013;22(127):58-65.
16. Southern KW. Atypical cystic fibrosis. European Respiratory Monograph. 2006(35):38-49.
17. Paranjape SM, Zeitlin PL. Atypical cystic fibrosis and CFTR-related diseases. Clinical reviews in allergy & immunology. 2008;35(3):116-23.
18. Drumm ML, Konstan MW, Schluchter MD, Handler A, Pace R, Zou F, et al. Genetic modifiers of lung disease in cystic fibrosis. The New England journal of medicine. 2005;353(14):1443-53.
19. Nadeau JH. Modifier genes in mice and humans. Nature reviews Genetics. 2001;2(3):165-74.
20. Cutting GR. Modifier genes in Mendelian disorders: the example of cystic fibrosis. Annals of the New York Academy of Sciences. 2010;1214:57-69.
21. Wright FA, Strug LJ, Doshi VK, Commander CW, Blackman SM, Sun L, et al. Genome-wide association and linkage identify modifier loci of lung disease severity in cystic fibrosis at 11p13 and 20q13.2. Nature genetics. 2011;43(6):539-46.
22. Blackman SM, Hsu S, Ritter SE, Naughton KM, Wright FA, Drumm ML, et al. A susceptibility gene for type 2 diabetes confers substantial risk for diabetes complicating cystic fibrosis. Diabetologia. 2009;52(9):1858-65.
23. Marson FA, Bertuzzo CS, Ribeiro AF, Ribeiro JD. Polymorphisms in the glutathione pathway modulate cystic fibrosis severity: a cross-sectional study. BMC medical genetics. 2014;15:27.
24. Li W, Soave D, Miller MR, Keenan K, Lin F, Gong J, et al. Unraveling the complex genetic model for cystic fibrosis: pleiotropic effects of modifier genes on early cystic fibrosis-related morbidities. Human genetics. 2014;133(2):151-61.
25. Ratner D, Mueller C. Immune responses in Cystic Fibrosis; are they intrinsically defective? American journal of respiratory cell and molecular biology. 2012;46(6):715-22.

26. Armstrong DS, Hook SM, Jamsen KM, Nixon GM, Carzino R, Carlin JB, et al. Lower airway inflammation in infants with cystic fibrosis detected by newborn screening. Pediatric pulmonology. 2005;40(6):500-10.
27. Sly PD, Brennan S, Gangell C, de Klerk N, Murray C, Mott L, et al. Lung disease at diagnosis in infants with cystic fibrosis detected by newborn screening. American journal of respiratory and critical care medicine. 2009;180(2):146-52.
28. Stafler P, Davies JC, Balfour-Lynn IM, Rosenthal M, Bush A. Bronchoscopy in cystic fibrosis infants diagnosed by newborn screening. Pediatric pulmonology. 2011;46(7):696-700.
29. Cone RA. Barrier properties of mucus. Advanced drug delivery reviews. 2009;61(2):75-85.
30. Henke MO, John G, Rheineck C, Chillappagari S, Naehrlich L, Rubin BK. Serine proteases degrade airway mucins in cystic fibrosis. Infection and immunity. 2011;79(8):3438-44.
31. von Bredow C, Birrer P, Griese M. Surfactant protein A and other bronchoalveolar lavage fluid proteins are altered in cystic fibrosis. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 2001;17(4):716-22.
32. Rogan MP, Taggart CC, Greene CM, Murphy PG, O'Neill SJ, McElvaney NG. Loss of microbicidal activity and increased formation of biofilm due to decreased lactoferrin activity in patients with cystic fibrosis. The Journal of infectious diseases. 2004;190(7):1245-53.
33. Moskwa P, Lorentzen D, Excoffon KJ, Zabner J, McCray PB, Jr., Nauseef WM, et al. A novel host defense system of airways is defective in cystic fibrosis. American journal of respiratory and critical care medicine. 2007;175(2):174-83.
34. Virella-Lowell I, Herlihy JD, Liu B, Lopez C, Cruz P, Muller C, et al. Effects of CFTR, interleukin-10, and *Pseudomonas aeruginosa* on gene expression profiles in a CF bronchial epithelial cell Line. Molecular therapy : the journal of the American Society of Gene Therapy. 2004;10(3):562-73.
35. Chen J, Jiang XH, Chen H, Guo JH, Tsang LL, Yu MK, et al. CFTR negatively regulates cyclooxygenase-2-PGE(2) positive feedback loop in inflammation. Journal of cellular physiology. 2012;227(6):2759-66.

36. Gao L, Kim KJ, Yankaskas JR, Forman HJ. Abnormal glutathione transport in cystic fibrosis airway epithelia. The American journal of physiology. 1999;277(1 Pt 1):L113-8.
37. Balghi H, Robert R, Rappaz B, Zhang X, Wohlhuter-Haddad A, Evagelidis A, et al. Enhanced Ca^{2+} entry due to Orai1 plasma membrane insertion increases IL-8 secretion by cystic fibrosis airways. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2011;25(12):4274-91.
38. Downey DG, Bell SC, Elborn JS. Neutrophils in cystic fibrosis. Thorax. 2009;64(1):81-8.
39. Cowburn AS, Condliffe AM, Farahi N, Summers C, Chilvers ER. Advances in neutrophil biology: clinical implications. Chest. 2008;134(3):606-12.
40. Painter RG, Valentine VG, Lanson NA, Jr., Leidal K, Zhang Q, Lombard G, et al. CFTR Expression in human neutrophils and the phagolysosomal chlorination defect in cystic fibrosis. Biochemistry. 2006;45(34):10260-9.
41. Adib-Conquy M, Pedron T, Petit-Bertron AF, Tabary O, Corvol H, Jacquot J, et al. Neutrophils in cystic fibrosis display a distinct gene expression pattern. Molecular medicine. 2008;14(1-2):36-44.
42. Dalli J, Rosignoli G, Hayhoe RP, Edelman A, Perretti M. CFTR inhibition provokes an inflammatory response associated with an imbalance of the annexin A1 pathway. The American journal of pathology. 2010;177(1):176-86.
43. Di A, Brown ME, Deriy LV, Li C, Szeto FL, Chen Y, et al. CFTR regulates phagosome acidification in macrophages and alters bactericidal activity. Nature cell biology. 2006;8(9):933-44.
44. Haggie PM, Verkman AS. Cystic fibrosis transmembrane conductance regulator-independent phagosomal acidification in macrophages. The Journal of biological chemistry. 2007;282(43):31422-8.
45. Vandivier RW, Richens TR, Horstmann SA, deCathelineau AM, Ghosh M, Reynolds SD, et al. Dysfunctional cystic fibrosis transmembrane conductance regulator inhibits phagocytosis of apoptotic cells with proinflammatory consequences. American journal of physiology Lung cellular and molecular physiology. 2009;297(4):L677-86.
46. Knight RA, Kollnberger S, Madden B, Yacoub M, Hodson ME. Defective antigen presentation by lavage cells from terminal patients with cystic fibrosis. Clinical and experimental immunology. 1997;107(3):542-7.

47. Regamey N, Tsartsali L, Hilliard TN, Fuchs O, Tan HL, Zhu J, et al. Distinct patterns of inflammation in the airway lumen and bronchial mucosa of children with cystic fibrosis. Thorax. 2012;67(2):164-70.
48. Hubeau C, Lorenzato M, Couetil JP, Hubert D, Dusser D, Puchelle E, et al. Quantitative analysis of inflammatory cells infiltrating the cystic fibrosis airway mucosa. Clinical and experimental immunology. 2001;124(1):69-76.
49. Moser C, Jensen PO. Adaptive Immune Responses and Biofilm Infections. Biofilm Infections. 2011:201-14.
50. Hartl D, Griese M, Kappler M, Zissel G, Reinhardt D, Rebhan C, et al. Pulmonary T(H)2 response in *Pseudomonas aeruginosa*-infected patients with cystic fibrosis. The Journal of allergy and clinical immunology. 2006;117(1):204-11.
51. Moser C, Kjaergaard S, Pressler T, Kharazmi A, Koch C, Hoiby N. The immune response to chronic *Pseudomonas aeruginosa* lung infection in cystic fibrosis patients is predominantly of the Th2 type. APMIS : acta pathologica, microbiologica, et immunologica Scandinavica. 2000;108(5):329-35.
52. Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, et al. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. The Journal of experimental medicine. 2005;201(2):233-40.
53. Dubin PJ, McAllister F, Kolls JK. Is cystic fibrosis a Th17 disease? Inflamm Res. 2007;56:221-7.
54. Kushwah R, Gagnon S, Sweezey NB. Intrinsic predisposition of naive cystic fibrosis T cells to differentiate towards a Th17 phenotype. Respiratory research. 2013;14:138.
55. Tan HL, Regamey N, Brown S, Bush A, Lloyd CM, Davies JC. The Th17 pathway in cystic fibrosis lung disease. American journal of respiratory and critical care medicine. 2011;184(2):252-8.
56. Tiringer K, Treis A, Fucik P, Gona M, Gruber S, Renner S, et al. A Th17- and Th2-skewed cytokine profile in cystic fibrosis lungs represents a potential risk factor for *Pseudomonas aeruginosa* infection. American journal of respiratory and critical care medicine. 2013;187(6):621-9.
57. Hartl D, Gaggar A, Bruscia E, Hector A, Marcos V, Jung A, et al. Innate immunity in cystic fibrosis lung disease. Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society. 2012;11(5):363-82.

58. Rieber N, Hector A, Carevic M, Hartl D. Current concepts of immune dysregulation in cystic fibrosis. The international journal of biochemistry & cell biology. 2014;52:108-12.
59. Josefowicz SZ, Lu LF, Rudensky AY. Regulatory T cells: mechanisms of differentiation and function. Annual Review of Immunology. 2012;30:531-64.
60. Li XN, Pan X, Qiu D. Imbalances of Th17 and Treg cells and their respective cytokines in COPD patients by disease stage. International Journal of Clinical and Experimental Medicine. 2014;7(12):5324-9.
61. Robinson DS. Regulatory T cells and asthma. Clinical and Experimental Allergy. 2009;39(9):1314-23.
62. Kessel A, Bamberger E, Masalha M, Toubi E. The role of T regulatory cells in human sepsis. Journal of Autoimmunity. 2009;32(3-4):211-5.
63. Kursar M, Koch M, Mittrucker HW, Nouailles G, Bonhagen K, Kamradt T, et al. Cutting Edge: Regulatory T cells prevent efficient clearance of Mycobacterium tuberculosis. Journal of immunology. 2007;178(5):2661-5.
64. Anil N, Singh M. CD4(+)CD25(high) FOXP3(+) regulatory T cells correlate with FEV1 in North Indian children with cystic fibrosis. Immunological Investigations. 2014;43(6):535-43.
65. Hector A, Schafer H, Poschel S, Fischer A, Fritzsching B, Ralhan A, et al. Regulatory T-cell impairment in cystic fibrosis patients with chronic pseudomonas infection. American journal of respiratory and critical care medicine. 2015;191(8):914-23.
66. Ito M, Kojiro N, Ikeda T, Ito T, Funada J, Kokubu T. Increased proportions of peripheral blood gamma delta T cells in patients with pulmonary tuberculosis. Chest. 1992;102(1):195-7.
67. Sutton CE, Mielke LA, Mills KH. IL-17-producing gammadelta T cells and innate lymphoid cells. European journal of immunology. 2012;42(9):2221-31.
68. Raga S, Julia MR, Crespi C, Figuerola J, Martinez N, Mila J, et al. Gammadelta T lymphocytes from cystic fibrosis patients and healthy donors are high TNF-alpha and IFN-gamma-producers in response to *Pseudomonas aeruginosa*. Respiratory research. 2003;4:9.
69. Gold MC, Lewinsohn DM. Co-dependents: MR1-restricted MAIT cells and their antimicrobial function. Nature reviews Microbiology. 2013;11(1):14-9.

70. Le Bourhis L, Martin E, Peguillet I, Guihot A, Froux N, Core M, et al. Antimicrobial activity of mucosal-associated invariant T cells. Nature immunology. 2010;11(8):701-8.
71. Lanier LL. Shades of grey--the blurring view of innate and adaptive immunity. Nature reviews Immunology. 2013;13(2):73-4.
72. Meierovics A, Yankelevich WJ, Cowley SC. MAIT cells are critical for optimal mucosal immune responses during in vivo pulmonary bacterial infection. Proceedings of the National Academy of Sciences of the United States of America. 2013;110(33):E3119-28.
73. Ranganathan SC, Parsons F, Gangell C, Brennan S, Stick SM, Sly PD, et al. Evolution of pulmonary inflammation and nutritional status in infants and young children with cystic fibrosis. Thorax. 2011;66(5):408-13.
74. Tunney MM, Field TR, Moriarty TF, Patrick S, Doering G, Muhlebach MS, et al. Detection of anaerobic bacteria in high numbers in sputum from patients with cystic fibrosis. American journal of respiratory and critical care medicine. 2008;177(9):995-1001.
75. Emerson J, McNamara S, Buccat AM, Worrell K, Burns JL. Changes in cystic fibrosis sputum microbiology in the United States between 1995 and 2008. Pediatric pulmonology. 2010;45(4):363-70.
76. Lim WS, Baudouin SV, George RC, Hill AT, Jamieson C, Le Jeune I, et al. BTS guidelines for the management of community acquired pneumonia in adults: update 2009. Thorax. 2009;64 Suppl 3:iii1-55.
77. Mandell LA, Wunderink RG, Anzueto A, Bartlett JG, Campbell GD, Dean NC, et al. Infectious Diseases Society of America/American Thoracic Society consensus guidelines on the management of community-acquired pneumonia in adults. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2007;44 Suppl 2:S27-72.
78. Aaron SD, Vandemheen KL, Ferris W, Fergusson D, Tullis E, Haase D, et al. Combination antibiotic susceptibility testing to treat exacerbations of cystic fibrosis associated with multiresistant bacteria: a randomised, double-blind, controlled clinical trial. Lancet. 2005;366(9484):463-71.
79. Rogers GB, Carroll MP, Serisier DJ, Hockey PM, Jones G, Bruce KD. Characterization of bacterial community diversity in cystic fibrosis lung infections by

use of 16s ribosomal DNA terminal restriction fragment length polymorphism profiling. Journal of clinical microbiology. 2004;42(11):5176-83.

80. Rogers GB, Daniels TW, Tuck A, Carroll MP, Connett GJ, David GJ, et al. Studying bacteria in respiratory specimens by using conventional and molecular microbiological approaches. BMC Pulmonary Medicine. 2009;9:14.
81. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The human microbiome project. Nature. 2007;449(7164):804-10.
82. Huang YJ, Lynch SV. The emerging relationship between the airway microbiota and chronic respiratory disease: clinical implications. Expert Review of Respiratory Medicine. 2011;5(6):809-21.
83. Cho I, Blaser MJ. The human microbiome: at the interface of health and disease. Nature reviews Genetics. 2012;13(4):260-70.
84. Cox MJ, Allgaier M, Taylor B, Baek MS, Huang YJ, Daly RA, et al. Airway microbiota and pathogen abundance in age-stratified cystic fibrosis patients. PloS one. 2010;5(6):e11044.
85. Zhao J, Schloss PD, Kalikin LM, Carmody LA, Foster BK, Petrosino JF, et al. Decade-long bacterial community dynamics in cystic fibrosis airways. Proceedings of the National Academy of Sciences of the United States of America. 2012;109(15):5809-14.
86. Willner D, Haynes MR, Furlan M, Schmieder R, Lim YW, Rainey PB, et al. Spatial distribution of microbial communities in the cystic fibrosis lung. ISME Journal. 2012;6(2):471-4.
87. Goddard AF, Staudinger BJ, Dowd SE, Joshi-Datar A, Wolcott RD, Aitken ML, et al. Direct sampling of cystic fibrosis lungs indicates that DNA-based analyses of upper-airway specimens can misrepresent lung microbiota. Proceedings of the National Academy of Sciences of the United States of America. 2012;109(34):13769-74.
88. Kobayashi H, Kobayashi O, Kawai S. Pathogenesis and clinical manifestations of chronic colonization by *Pseudomonas aeruginosa* and its biofilms in the airway tract. Journal of infection and chemotherapy : official journal of the Japan Society of Chemotherapy. 2009;15(3):125-42.
89. Rau MH, Hansen SK, Johansen HK, Thomsen LE, Workman CT, Nielsen KF, et al. Early adaptive developments of *Pseudomonas aeruginosa* after the transition from life in the environment to persistent colonization in the airways of human cystic fibrosis hosts. Environmental microbiology. 2010;12(6):1643-58.

90. Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, et al. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature. 2000;406(6799):959-64.
91. Trautmann M, Lepper PM, Haller M. Ecology of *Pseudomonas aeruginosa* in the intensive care unit and the evolving role of water outlets as a reservoir of the organism. American journal of infection control. 2005;33(5 Suppl 1):S41-9.
92. Estahbanati HK, Kashani PP, Ghanaatpisheh F. Frequency of *Pseudomonas aeruginosa* serotypes in burn wound infections and their resistance to antibiotics. Burns : journal of the International Society for Burn Injuries. 2002;28(4):340-8.
93. Murphy TF. *Pseudomonas aeruginosa* in adults with chronic obstructive pulmonary disease. Current opinion in pulmonary medicine. 2009;15(2):138-42.
94. Emerson J, Rosenfeld M, McNamara S, Ramsey B, Gibson RL. *Pseudomonas aeruginosa* and other predictors of mortality and morbidity in young children with cystic fibrosis. Pediatric pulmonology. 2002;34(2):91-100.
95. Hassett DJ, Korfhagen TR, Irvin RT, Schurr MJ, Sauer K, Lau GW, et al. *Pseudomonas aeruginosa* biofilm infections in cystic fibrosis: insights into pathogenic processes and treatment strategies. Expert opinion on therapeutic targets. 2010;14(2):117-30.
96. Bjarnsholt T, Jensen PO, Fiandaca MJ, Pedersen J, Hansen CR, Andersen CB, et al. *Pseudomonas aeruginosa* biofilms in the respiratory tract of cystic fibrosis patients. Pediatric pulmonology. 2009;44(6):547-58.
97. Klausen M, Heydorn A, Ragas P, Lambertsen L, Aaes-Jorgensen A, Molin S, et al. Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. Molecular microbiology. 2003;48(6):1511-24.
98. Klausen M, Aaes-Jorgensen A, Molin S, Tolker-Nielsen T. Involvement of bacterial migration in the development of complex multicellular structures in *Pseudomonas aeruginosa* biofilms. Molecular microbiology. 2003;50(1):61-8.
99. Chen X, Stewart PS. Role of electrostatic interactions in cohesion of bacterial biofilms. Applied microbiology and biotechnology. 2002;59(6):718-20.
100. Bollinger N, Hassett DJ, Iglewski BH, Costerton JW, McDermott TR. Gene expression in *Pseudomonas aeruginosa*: evidence of iron override effects on quorum sensing and biofilm-specific gene regulation. Journal of bacteriology. 2001;183(6):1990-6.

101. Duan K, Surette MG. Environmental regulation of *Pseudomonas aeruginosa* PAO1 Las and Rhl quorum-sensing systems. Journal of bacteriology. 2007;189(13):4827-36.
102. Oglesby AG, Farrow JM, 3rd, Lee JH, Tomaras AP, Greenberg EP, Pesci EC, et al. The influence of iron on *Pseudomonas aeruginosa* physiology: a regulatory link between iron and quorum sensing. The Journal of biological chemistry. 2008;283(23):15558-67.
103. Diggle SP, Matthijs S, Wright VJ, Fletcher MP, Chhabra SR, Lamont IL, et al. The *Pseudomonas aeruginosa* 4-quinolone signal molecules HHQ and PQS play multifunctional roles in quorum sensing and iron entrapment. Chemistry & biology. 2007;14(1):87-96.
104. O'Carroll MR, Syrmis MW, Wainwright CE, Greer RM, Mitchell P, Coulter C, et al. Clonal strains of *Pseudomonas aeruginosa* in paediatric and adult cystic fibrosis units. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 2004;24(1):101-6.
105. Scott FW, Pitt TL. Identification and characterization of transmissible *Pseudomonas aeruginosa* strains in cystic fibrosis patients in England and Wales. Journal of medical microbiology. 2004;53(Pt 7):609-15.
106. Aaron SD, Vandemheen KL, Ramotar K, Giesbrecht-Lewis T, Tullis E, Freitag A, et al. Infection with transmissible strains of *Pseudomonas aeruginosa* and clinical outcomes in adults with cystic fibrosis. JAMA : the journal of the American Medical Association. 2010;304(19):2145-53.
107. Kidd TJ, Ramsay KA, Hu H, Marks GB, Wainwright CE, Bye PT, et al. Shared *Pseudomonas aeruginosa* genotypes are common in Australian cystic fibrosis centres. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 2013;41(5):1091-100.
108. Al-Aloul M, Crawley J, Winstanley C, Hart CA, Ledson MJ, Walshaw MJ. Increased morbidity associated with chronic infection by an epidemic *Pseudomonas aeruginosa* strain in CF patients. Thorax. 2004;59(4):334-6.
109. Griffiths AL, Jamsen K, Carlin JB, Grimwood K, Carzino R, Robinson PJ, et al. Effects of segregation on an epidemic *Pseudomonas aeruginosa* strain in a cystic fibrosis clinic. American journal of respiratory and critical care medicine. 2005;171(9):1020-5.

110. Wientzen R, Prestidge CB, Kramer RI, McCracken GH, Nelson JD. Acute pulmonary exacerbations in cystic fibrosis. A double-blind trial of tobramycin and placebo therapy. American Journal of Diseases of Children. 1980;134(12):1134-8.
111. Gold R, Carpenter S, Heurter H, Corey M, Levison H. Randomized trial of ceftazidime versus placebo in the management of acute respiratory exacerbations in patients with cystic fibrosis. Journal of Pediatrics. 1987;111(6 Pt 1):907-13.
112. Smyth A, Elborn JS. Exacerbations in cystic fibrosis: 3--Management. Thorax. 2008;63(2):180-4.
113. Valerius NH, Koch C, Hoiby N. Prevention of chronic *Pseudomonas aeruginosa* colonisation in cystic fibrosis by early treatment. Lancet. 1991;338(8769):725-6.
114. Wiesemann HG, Steinkamp G, Ratjen F, Bauernfeind A, Przyklenk B, Doring G, et al. Placebo-controlled, double-blind, randomized study of aerosolized tobramycin for early treatment of *Pseudomonas aeruginosa* colonization in cystic fibrosis. Pediatric pulmonology. 1998;25(2):88-92.
115. Ratjen F, Doring G, Nikolaizik WH. Effect of inhaled tobramycin on early *Pseudomonas aeruginosa* colonisation in patients with cystic fibrosis. Lancet. 2001;358(9286):983-4.
116. Ratjen F, Munck A, Kho P, Angyalosi G, Group ES. Treatment of early *Pseudomonas aeruginosa* infection in patients with cystic fibrosis: the ELITE trial. Thorax. 2010;65(4):286-91.
117. Wainwright C, Vidmer S, Armstrong D, Byrnes C, Carlin JB, Cheney J, et al. Effect of Bronchoalveolar Lavage-Directed Therapy on *Pseudomonas aeruginosa* Infection and Structural Lung Injury in Children With Cystic Fibrosis: A Randomized Trial. JAMA : the journal of the American Medical Association. 2011(In Press).
118. Liou TG, Adler FR, Fitzsimmons SC, Cahill BC, Hibbs JR, Marshall BC. Predictive 5-year survivorship model of cystic fibrosis. American Journal of Epidemiology. 2001;153(4):345-52.
119. Sanders DB, Bittner RC, Rosenfeld M, Redding GJ, Goss CH. Pulmonary exacerbations are associated with subsequent FEV_1 decline in both adults and children with cystic fibrosis. Pediatric pulmonology. 2011;46(4):393-400.
120. Britto MT, Kotagal UR, Hornung RW, Atherton HD, Tsevat J, Wilmott RW. Impact of recent pulmonary exacerbations on quality of life in patients with cystic fibrosis. Chest. 2002;121(1):64-72.

121. Lieu TA, Ray GT, Farmer G, Shay GF. The cost of medical care for patients with cystic fibrosis in a health maintenance organization. Pediatrics. 1999;103(6):e72.
122. Retsch-Bogart GZ, Quittner AL, Gibson RL, Oermann CM, McCoy KS, Montgomery AB, et al. Efficacy and safety of inhaled aztreonam lysine for airway pseudomonas in cystic fibrosis. Chest. 2009;135(5):1223-32.
123. Oermann CM, Retsch-Bogart GZ, Quittner AL, Gibson RL, McCoy KS, Montgomery AB, et al. An 18-month study of the safety and efficacy of repeated courses of inhaled aztreonam lysine in cystic fibrosis. Pediatric pulmonology. 2010;45(11):1121-34.
124. Remmington T, Jahnke N, Harkensee C. Oral anti-pseudomonal antibiotics for cystic fibrosis. Cochrane Database of Systematic Reviews. 2007(3):CD005405.
125. Guidelines for the management of adults with hospital-acquired, ventilator-associated, and healthcare-associated pneumonia. American journal of respiratory and critical care medicine. 2005;171(4):388-416.
126. Elphick HE, Tan A. Single versus combination intravenous antibiotic therapy for people with cystic fibrosis. Cochrane Database of Systematic Reviews. 2005(2):CD002007.
127. Flume PA, Mogayzel PJ, Jr., Robinson KA, Goss CH, Rosenblatt RL, Kuhn RJ, et al. Cystic fibrosis pulmonary guidelines: treatment of pulmonary exacerbations. American journal of respiratory and critical care medicine. 2009;180(9):802-8.
128. Yang I, Kim S, Bell S. Antibiotics in COPD, bronchiectasis, and cystic fibrosis. In: Gibson PG, editor. Evidence-based respiratory medicine. Oxford: Blackwell Publishing; 2005. p. 389-413.
129. Antibiotic Consensus Group. Antibiotic Treatment of Cystic Fibrosis: Report of the UK Cystic Fibrosis Trust antibiotic working group. 3rd ed: Cystic Fibrosis Trust; 2009.
130. Limited TG. Therapeutic Guidelines: antibiotics. Expert group for respiratory infections (14). Melbourne; 2010.
131. Plummer A, Wildman M. Duration of intravenous antibiotic therapy in people with cystic fibrosis. Cochrane Database of Systematic Reviews. 2011(1):CD006682.
132. Falagas ME, Avgeri SG, Matthaiou DK, Dimopoulos G, Siempos, II. Short- versus long-duration antimicrobial treatment for exacerbations of chronic bronchitis: a meta-analysis. Journal of Antimicrobial Chemotherapy. 2008;62(3):442-50.
133. Chastre J, Wolff M, Fagon JY, Chevret S, Thomas F, Wermert D, et al. Comparison of 8 vs 15 days of antibiotic therapy for ventilator-associated pneumonia in adults: a

randomized trial. JAMA : the journal of the American Medical Association. 2003;290(19):2588-98.

134. Bell SC, Bowerman AM, Nixon LE, Macdonald IA, Elborn JS, Shale DJ. Metabolic and inflammatory responses to pulmonary exacerbation in adults with cystic fibrosis. European journal of clinical investigation. 2000;30(6):553-9.

135. Collaco JM, Green DM, Cutting GR, Naughton KM, Mogayzel PJ, Jr. Location and duration of treatment of cystic fibrosis respiratory exacerbations do not affect outcomes. American journal of respiratory and critical care medicine. 2010;182(9):1137-43.

136. Tunney MM, Klem ER, Fodor AA, Gilpin DF, Moriarty TF, McGrath SJ, et al. Use of culture and molecular analysis to determine the effect of antibiotic treatment on microbial community diversity and abundance during exacerbation in patients with cystic fibrosis. Thorax. 2011;66(7):579-84.

137. Daniels TW, Rogers GB, Stressmann FA, van der Gast CJ, Bruce KD, Jones GR, et al. Impact of antibiotic treatment for pulmonary exacerbations on bacterial diversity in cystic fibrosis. Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society. 2013;12(1):22-8.

138. Chua AC, Graham RM, Trinder D, Olynyk JK. The regulation of cellular iron metabolism. Critical Reviews in Clinical Laboratory Sciences. 2007;44(5-6):413-59.

139. Koppenol WH. The centennial of the Fenton reaction. Free radical biology & medicine. 1993;15(6):645-51.

140. Krause A, Neitz S, Magert HJ, Schulz A, Forssmann WG, Schulz-Knappe P, et al. LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity. FEBS Letters. 2000;480(2-3):147-50.

141. Park CH, Valore EV, Waring AJ, Ganz T. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. The Journal of biological chemistry. 2001;276(11):7806-10.

142. Pigeon C, Ilyin G, Courselaud B, Leroyer P, Turlin B, Brissot P, et al. A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. The Journal of biological chemistry. 2001;276(11):7811-9.

143. Ganz T. Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation. Blood. 2003;102(3):783-8.

144. Ganz T. Hepcidin and iron regulation, 10 years later. Blood. 2011;117(17):4425-33.

145. Barton JC. Hemochromatosis and iron overload: from bench to clinic. The American journal of the medical sciences. 2013;346(5):403-12.
146. Olynyk JK, Cullen DJ, Aquilia S, Rossi E, Summerville L, Powell LW. A population-based study of the clinical expression of the hemochromatosis gene. The New England journal of medicine. 1999;341(10):718-24.
147. Pasricha SR, Flecknoe-Brown SC, Allen KJ, Gibson PR, McMahon LP, Olynyk JK, et al. Diagnosis and management of iron deficiency anaemia: a clinical update. The Medical journal of Australia. 2010;193(9):525-32.
148. Wish JB. Assessing iron status: beyond serum ferritin and transferrin saturation. Clinical journal of the American Society of Nephrology : CJASN. 2006;1 Suppl 1:S4-8.
149. Skikne BS, Punnonen K, Caldron PH, Bennett MT, Rehu M, Gasior GH, et al. Improved differential diagnosis of anemia of chronic disease and iron deficiency anemia: a prospective multicenter evaluation of soluble transferrin receptor and the sTfR/log ferritin index. American journal of hematology. 2011;86(11):923-7.
150. Skikne BS, Flowers CH, Cook JD. Serum transferrin receptor: a quantitative measure of tissue iron deficiency. Blood. 1990;75(9):1870-6.
151. Punnonen K, Irjala K, Rajamaki A. Serum transferrin receptor and its ratio to serum ferritin in the diagnosis of iron deficiency. Blood. 1997;89(3):1052-7.
152. Suominen P, Punnonen K, Rajamaki A, Irjala K. Serum transferrin receptor and transferrin receptor-ferritin index identify healthy subjects with subclinical iron deficits. Blood. 1998;92(8):2934-9.
153. Aust AE, Ball JC, Hu AA, Lighty JS, Smith KR, Straccia AM, et al. Particle characteristics responsible for effects on human lung epithelial cells. Research report. 2002(110):1-65; discussion 7-76.
154. Turi JL, Yang F, Garrick MD, Piantadosi CA, Ghio AJ. The iron cycle and oxidative stress in the lung. Free radical biology & medicine. 2004;36(7):850-7.
155. Legrand D, Mazurier J. A critical review of the roles of host lactoferrin in immunity. Biometals. 2010;23(3):365-76.
156. Cole AM, Waring AJ. The role of defensins in lung biology and therapy. American Journal of Respiratory and Critical Care Medicine. 2002;1(4):249-59.
157. Ghio AJ. Disruption of iron homeostasis and lung disease. Biochimica et biophysica acta. 2009;1790(7):731-9.

158. Yang F, Wang X, Haile DJ, Piantadosi CA, Ghio AJ. Iron increases expression of iron-export protein MTP1 in lung cells. American journal of physiology Lung cellular and molecular physiology. 2002;283(5):L932-9.
159. Ghio AJ, Turi JL, Yang F, Garrick LM, Garrick MD. Iron homeostasis in the lung. Biological Research. 2006;39(1):67-77.
160. Turi JL, Wang X, McKie AT, Nozik-Grayck E, Mamo LB, Crissman K, et al. Duodenal cytochrome b: a novel ferrireductase in airway epithelial cells. American journal of physiology Lung cellular and molecular physiology. 2006;291(2):L272-80.
161. Ghio AJ, Nozik-Grayck E, Turi J, Jaspers I, Mercatante DR, Kole R, et al. Superoxide-dependent iron uptake: a new role for anion exchange protein 2. American journal of respiratory cell and molecular biology. 2003;29(6):653-60.
162. Forbes JR, Gros P. Divalent-metal transport by NRAMP proteins at the interface of host-pathogen interactions. Trends in Microbiology. 2001;9(8):397-403.
163. Ghio AJ, Carter JD, Richards JH, Richer LD, Grissom CK, Elstad MR. Iron and iron-related proteins in the lower respiratory tract of patients with acute respiratory distress syndrome. Critical Care Medicine. 2003;31(2):395-400.
164. Sharkey RA, Donnelly SC, Connelly KG, Robertson CE, Haslett C, Repine JE. Initial serum ferritin levels in patients with multiple trauma and the subsequent development of acute respiratory distress syndrome. American journal of respiratory and critical care medicine. 1999;159(5 Pt 1):1506-9.
165. Lagan AL, Quinlan GJ, Mumby S, Melley DD, Goldstraw P, Bellingan GJ, et al. Variation in iron homeostasis genes between patients with ARDS and healthy control subjects. Chest. 2008;133(6):1302-11.
166. Gangaidzo IT, Moyo VM, Mvundura E, Aggrey G, Murphree NL, Khumalo H, et al. Association of pulmonary tuberculosis with increased dietary iron. The Journal of infectious diseases. 2001;184(7):936-9.
167. Gifford AH, Alexandru DM, Li Z, Dorman DB, Moulton LA, Price KE, et al. Iron supplementation does not worsen respiratory health or alter the sputum microbiome in cystic fibrosis. Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society. 2014;13(3):311-8.
168. Collins JF, Wessling-Resnick M, Knutson MD. Hepcidin regulation of iron transport. The Journal of nutrition. 2008;138(11):2284-8.

169. Reid DW, Withers NJ, Francis L, Wilson JW, Kotsimbos TC. Iron deficiency in cystic fibrosis: relationship to lung disease severity and chronic *Pseudomonas aeruginosa* infection. Chest. 2002;121(1):48-54.
170. Keevil B, Rowlands D, Burton I, Webb AK. Assessment of iron status in cystic fibrosis patients. Annals of clinical biochemistry. 2000;37 (Pt 5):662-5.
171. Uijterschout L, Swinkels DW, Akkermans MD, Zandstra TThe value of soluble transferrin receptor and hepcidin in the assessment of iro, Nuijsink M, Hendriks D, et al. n status in children with cystic fibrosis. Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society. 2014;13(6):639-44.
172. Hoo ZH, Wildman MJ. Intravenous iron among cystic fibrosis patients. Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society. 2012;11(6):560-2.
173. Devaney J, Maher M, Smith T, Houghton JA, Glennon M. *HFE* alleles in an Irish cystic fibrosis population. Genetic testing. 2003;7(2):155-8.
174. Pratap U, Quinn S, Blizzard LB, Reid DW. Population-based study of cystic fibrosis disease severity and haemochromatosis gene mutations. Respirology. 2010;15(1):141-9.
175. Rohlfs EM, Shaheen NJ, Silverman LM. Is the hemochromatosis gene a modifier locus for cystic fibrosis? Genetic testing. 1998;2(1):85-8.
176. Stites SW, Plautz MW, Bailey K, O'Brien-Ladner AR, Wesselius LJ. Increased concentrations of iron and isoferritins in the lower respiratory tract of patients with stable cystic fibrosis. American journal of respiratory and critical care medicine. 1999;160(3):796-801.
177. Reid DW, Lam QT, Schneider H, Walters EH. Airway iron and iron-regulatory cytokines in cystic fibrosis. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 2004;24(2):286-91.
178. Stites SW, Walters B, O'Brien-Ladner AR, Bailey K, Wesselius LJ. Increased iron and ferritin content of sputum from patients with cystic fibrosis or chronic bronchitis. Chest. 1998;114(3):814-9.
179. Reid DW, Carroll V, O'May C, Champion A, Kirov SM. Increased airway iron as a potential factor in the persistence of *Pseudomonas aeruginosa* infection in cystic fibrosis. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 2007;30(2):286-92.

180. Moreau-Marquis S, Bomberger JM, Anderson GG, Swiatecka-Urban A, Ye S, O'Toole GA, et al. The DeltaF508-*CFTR* mutation results in increased biofilm formation by *Pseudomonas aeruginosa* by increasing iron availability. American journal of physiology Lung cellular and molecular physiology. 2008;295(1):L25-37.
181. Flume PA, Mogayzel PJ, Jr., Robinson KA, Rosenblatt RL, Quittell L, Marshall BC, et al. Cystic fibrosis pulmonary guidelines: pulmonary complications: hemoptysis and pneumothorax. American journal of respiratory and critical care medicine. 2010;182(3):298-306.
182. Vasil ML, Ochsner UA. The response of *Pseudomonas aeruginosa* to iron: genetics, biochemistry and virulence. Molecular microbiology. 1999;34(3):399-413.
183. Ochsner UA, Johnson Z, Vasil ML. Genetics and regulation of two distinct haem-uptake systems, phu and has, in *Pseudomonas aeruginosa*. Microbiology. 2000;146 (Pt 1):185-98.
184. Letoffe S, Redeker V, Wandersman C. Isolation and characterization of an extracellular haem-binding protein from *Pseudomonas aeruginosa* that shares function and sequence similarities with the Serratia marcescens HasA haemophore. Molecular microbiology. 1998;28(6):1223-34.
185. Alontaga AY, Rodriguez JC, Schonbrunn E, Becker A, Funke T, Yukl ET, et al. Structural characterization of the hemophore HasAp from *Pseudomonas aeruginosa*: NMR spectroscopy reveals protein-protein interactions between Holo-HasAp and hemoglobin. Biochemistry. 2009;48(1):96-109.
186. Cornelis P. Iron uptake and metabolism in pseudomonads. Applied microbiology and biotechnology. 2010;86(6):1637-45.
187. Lamont IL, Konings AF, Reid DW. Iron acquisition by *Pseudomonas aeruginosa* in the lungs of patients with cystic fibrosis. Biometals. 2009;22(1):53-60.
188. Visca P, Imperi F, Lamont IL. Pyoverdine siderophores: from biogenesis to biosignificance. Trends in Microbiology. 2007;15(1):22-30.
189. Meyer JM, Stintzi A, De Vos D, Cornelis P, Tappe R, Taraz K, et al. Use of siderophores to type pseudomonads: the three *Pseudomonas aeruginosa* pyoverdine systems. Microbiology. 1997;143 (Pt 1):35-43.
190. Banin E, Vasil ML, Greenberg EP. Iron and *Pseudomonas aeruginosa* biofilm formation. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(31):11076-81.

191. Marshall B, Stintzi A, Gilmour C, Meyer JM, Poole K. Citrate-mediated iron uptake in *Pseudomonas aeruginosa*: involvement of the citrate-inducible FecA receptor and the FeoB ferrous iron transporter. Microbiology. 2009;155(Pt 1):305-15.
192. Cornelis P, Matthijs S, Van Oeffelen L. Iron uptake regulation in *Pseudomonas aeruginosa*. Biometals. 2009;22(1):15-22.
193. Prince RW, Cox CD, Vasil ML. Coordinate regulation of siderophore and exotoxin A production: molecular cloning and sequencing of the *Pseudomonas aeruginosa* fur gene. Journal of bacteriology. 1993;175(9):2589-98.
194. Wilderman PJ, Sowa NA, FitzGerald DJ, FitzGerald PC, Gottesman S, Ochsner UA, et al. Identification of tandem duplicate regulatory small RNAs in *Pseudomonas aeruginosa* involved in iron homeostasis. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(26):9792-7.
195. Vasil ML. How we learnt about iron acquisition in *Pseudomonas aeruginosa*: a series of very fortunate events. Biometals. 2007;20(3-4):587-601.
196. Smith DJ, Lamont IL, Anderson GJ, Reid DW. Targeting iron uptake to control *Pseudomonas aeruginosa* infections in cystic fibrosis. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 2013;42(6):1723-36.
197. Taccetti G, Bianchini E, Cariani L, Buzzetti R, Costantini D, Trevisan F, et al. Early antibiotic treatment for *Pseudomonas aeruginosa* eradication in patients with cystic fibrosis: a randomised multicentre study comparing two different protocols. Thorax. 2012;67(10):853-9.
198. Doring G. Eradication of *Pseudomonas aeruginosa* by early antibiotic treatment has been one of the major advances in the last decade for subjects with CF. Introduction. Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society. 2012;11(1):1.
199. Ratjen F. Treatment of early *Pseudomonas aeruginosa* infection in patients with cystic fibrosis. Current opinion in pulmonary medicine. 2006;12(6):428-32.
200. Cohen-Cymberknoh M, Shoseyov D, Kerem E. Managing cystic fibrosis: strategies that increase life expectancy and improve quality of life. American journal of respiratory and critical care medicine. 2011;183(11):1463-71.
201. Kerem E, Viviani L, Zolin A, Macneill S, Hatziagorou E, Ellemunter H, et al. Factors associated with FEV1 decline in cystic fibrosis: analysis of the data of the ECFS

Patient Registry. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 2014;43(1):125-33.

202. Doring G, Flume P, Heijerman H, Elborn JS, Consensus Study G. Treatment of lung infection in patients with cystic fibrosis: current and future strategies. Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society. 2012;11(6):461-79.

203. Boyle MP, Sabadosa KA, Quinton HB, Marshall BC, Schechter MS. Key findings of the US Cystic Fibrosis Foundation's clinical practice benchmarking project. BMJ quality & safety. 2014;23 Suppl 1:i15-i22.

204. Salvatore D, Buzzetti R, Baldo E, Furnari ML, Lucidi V, Manunza D, et al. An overview of international literature from cystic fibrosis registries. Part 4: update 2011. Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society. 2012;11(6):480-93.

205. Corey M, McLaughlin FJ, Williams M, Levison H. A comparison of survival, growth, and pulmonary function in patients with cystic fibrosis in Boston and Toronto. Journal of clinical epidemiology. 1988;41(6):583-91.

206. Johnson C, Butler SM, Konstan MW, Morgan W, Wohl ME. Factors influencing outcomes in cystic fibrosis: a center-based analysis. Chest. 2003;123(1):20-7.

207. Stern M. The use of a cystic fibrosis patient registry to assess outcomes and improve cystic fibrosis care in Germany. Current opinion in pulmonary medicine. 2011;17(6):473-7.

208. Stern M, Niemann N, Wiedemann B, Wenzlaff P, German CG. Benchmarking improves quality in cystic fibrosis care: a pilot project involving 12 centres. International journal for quality in health care : journal of the International Society for Quality in Health Care / ISQua. 2011;23(3):349-56.

209. Rogers GB, Carroll MP, Serisier DJ, Hockey PM, Jones G, Bruce KD. Characterization of bacterial community diversity in cystic fibrosis lung infections by use of 16s ribosomal DNA terminal restriction fragment length polymorphism profiling. Journal of clinical microbiology. 2004;42(11):5176-83.

210. Smith AL, Fiel SB, Mayer-Hamblett N, Ramsey B, Burns JL. Susceptibility testing of *Pseudomonas aeruginosa* isolates and clinical response to parenteral antibiotic administration: lack of association in cystic fibrosis. Chest. 2003;123(5):1495-502.

211. Lang BJ, Aaron SD, Ferris W, Hebert PC, MacDonald NE. Multiple combination bactericidal antibiotic testing for patients with cystic fibrosis infected with

multiresistant strains of *Pseudomonas aeruginosa*. American journal of respiratory and critical care medicine. 2000;162(6):2241-5.

212. Sanderson K, Wescombe L, Kirov SM, Champion A, Reid DW. Bacterial cyanogenesis occurs in the cystic fibrosis lung. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 2008;32(2):329-33.

213. Worlitzsch D, Tarran R, Ulrich M, Schwab U, Cekici A, Meyer KC, et al. Effects of reduced mucus oxygen concentration in airway Pseudomonas infections of cystic fibrosis patients. The Journal of clinical investigation. 2002;109(3):317-25.

214. Jomova K, Valko M. Advances in metal-induced oxidative stress and human disease. Toxicology. 2011;283(2-3):65-87.

215. Fraga CG. Relevance, essentiality and toxicity of trace elements in human health. Molecular aspects of medicine. 2005;26(4-5):235-44.

216. Gray RD, Duncan A, Noble D, Imrie M, O'Reilly DS, Innes JA, et al. Sputum trace metals are biomarkers of inflammatory and suppurative lung disease. Chest. 2010;137(3):635-41.

217. Glick R, Gilmour C, Tremblay J, Satanower S, Avidan O, Deziel E, et al. Increase in rhamnolipid synthesis under iron-limiting conditions influences surface motility and biofilm formation in *Pseudomonas aeruginosa*. Journal of bacteriology. 2010;192(12):2973-80.

218. Dieppois G, Ducret V, Caille O, Perron K. The transcriptional regulator CzcR modulates antibiotic resistance and quorum sensing in *Pseudomonas aeruginosa*. PloS one. 2012;7(5):e38148.

219. Werner JJ, Koren O, Hugenholtz P, DeSantis TZ, Walters WA, Caporaso JG, et al. Impact of training sets on classification of high-throughput bacterial 16s rRNA gene surveys. ISME Journal. 2012;6(1):94-103.

220. Dusseaux M, Martin E, Serriari N, Peguillet I, Premel V, Louis D, et al. Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161hi IL-17-secreting T cells. Blood. 2011;117(4):1250-9.

221. Benesova K, Vujic Spasic M, Schaefer SM, Stolte J, Baehr-Ivacevic T, Waldow K, et al. Hfe deficiency impairs pulmonary neutrophil recruitment in response to inflammation. PloS one. 2012;7(6):e39363.

222. Kerem E, Viviani L, Zolin A, MacNeill S, Hatziagorou E, Ellemunter H, et al. Factors associated with FEV_1 decline in cystic fibrosis: analysis of the ECFS patient registry.

The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 2014;43(1):125-33.

223. Fothergill JL, Walshaw MJ, Winstanley C. Transmissible strains of *Pseudomonas aeruginosa* in cystic fibrosis lung infections. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 2012;40(1):227-38.

224. Cystic Fibrosis Australia. Cystic Fibrosis in Australia 2009: 12th Annual Report from the Australian Cystic Fibrosis Data Registry. Sydney: Cystic Fibrosis Australia 2011, 2011.

225. Wang X, Dockery DW, Wypij D, Fay ME, Ferris BG, Jr. Pulmonary function between 6 and 18 years of age. Pediatric pulmonology. 1993;15(2):75-88.

226. Hankinson JL, Odencrantz JR, Fedan KB. Spirometric reference values from a sample of the general U.S. population. American journal of respiratory and critical care medicine. 1999;159(1):179-87.

227. Kuczmarski RJ, Ogden CL, Guo SS, Grummer-Strawn LM, Flegal KM, Mei Z, et al. 2000 CDC Growth Charts for the United States: methods and development. Vital and health statistics Series 11, Data from the national health survey. 2002(246):1-190.

228. Wolter JM, Kotsiou G, McCormack JG. Mixed morphotype testing of *Pseudomonas aeruginosa* cultures from cystic fibrosis patients. Journal of medical microbiology. 1995;42(3):220-4.

229. Breakpoint tables for interpretation of MICs and Zone Diameters, Version 1.3: European Committee on Antimicrobial Susceptibility Testing; 2011. Available from: http://www.eucast.org/clinical_breakpoints.

230. Performance Standards for Testing: Twenty-first Informational Supplement M100-S21.Antimicrobial Susceptibility Testing. Wayne, PA, USA: Clinical and Laboratory Standards Institute; 2011.

231. Bell SM, Pham JN, Fisher GT. Antibiotic susceptibility testing by the CDS method : a manual for medical and veterinary laboratories 2009. 5th ed. Sydney: The Antibiotic Reference Laboratory. South Eastern Area Laboratory Services; 2009.

232. Kidd TJ, Ramsay KA, Hu H, Bye PT, Elkins MR, Grimwood K, et al. Low rates of *Pseudomonas aeruginosa* misidentification in isolates from cystic fibrosis patients. Journal of Clinical Microbiology Journal of Clinical Microbiology. 2009;47(5):1503-9.

233. Saiman L, Mehar F, Niu WW, Neu HC, Shaw KJ, Miller G, et al. Antibiotic susceptibility of multiply resistant *Pseudomonas aeruginosa* isolated from patients with cystic fibrosis, including candidates for transplantation. Clinical Infectious Diseases. 1996;23(3):532-7.

234. Strateva T, Yordanov D. *Pseudomonas aeruginosa* - a phenomenon of bacterial resistance. Journal of medical microbiology. 2009;58(Pt 9):1133-48.

235. Harris A, Torres-Viera C, Venkataraman L, DeGirolami P, Samore M, Carmeli Y. Epidemiology and clinical outcomes of patients with multiresistant *Pseudomonas aeruginosa*. Clinical Infectious Diseases. 1999;28(5):1128-33.

236. Su S, Hassett DJ. Anaerobic *Pseudomonas aeruginosa* and other obligately anaerobic bacterial biofilms growing in the thick airway mucus of chronically infected cystic fibrosis patients: an emerging paradigm or "Old Hat"? Expert opinion on therapeutic targets. 2012;16(9):859-73.

237. Hurley MN, Ariff AH, Bertenshaw C, Bhatt J, Smyth AR. Results of antibiotic susceptibility testing do not influence clinical outcome in children with cystic fibrosis. Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society. 2012;11(4):288-92.

238. Lechtzin N, John M, Irizarry R, Merlo C, Diette GB, Boyle MP. Outcomes of adults with cystic fibrosis infected with antibiotic-resistant *Pseudomonas aeruginosa*. Respiration; international review of thoracic diseases. 2006;73(1):27-33.

239. Ren CL, Konstan MW, Yegin A, Rasouliyan L, Trzaskoma B, Morgan WJ, et al. Multiple antibiotic-resistant *Pseudomonas aeruginosa* and lung function decline in patients with cystic fibrosis. Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society. 2012;11(4):293-9.

240. Davies G, McShane D, Davies JC, Bush A. Multiresistant *Pseudomonas aeruginosa* in a pediatric cystic fibrosis center: natural history and implications for segregation. Pediatric pulmonology. 2003;35(4):253-6.

241. Prayle A, Smyth AR. Aminoglycoside use in cystic fibrosis: therapeutic strategies and toxicity. Current opinion in pulmonary medicine. 2010;16(6):604-10.

242. Sawicki GS, Sellers DE, Robinson WM. High treatment burden in adults with cystic fibrosis: challenges to disease self-management. Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society. 2009;8(2):91-6.

243. Cramer N, Wiehlmann L, Ciofu O, Tamm S, Hoiby N, Tummler B. Molecular epidemiology of chronic *Pseudomonas aeruginosa* airway infections in cystic fibrosis. PloS one. 2012;7(11):e50731.
244. Dalhoff A. Pharmacokinetics and pharmacodynamics of aerosolized antibacterial agents in chronically infected cystic fibrosis patients. Clinical microbiology reviews. 2014;27(4):753-82.
245. Flume PA, Robinson KA, O'Sullivan BP, Finder JD, Vender RL, Willey-Courand DB, et al. Cystic fibrosis pulmonary guidelines: airway clearance therapies. Respiratory care. 2009;54(4):522-37.
246. Cabana MD, Rand CS, Powe NR, Wu AW, Wilson MH, Abboud PA, et al. Why don't physicians follow clinical practice guidelines? A framework for improvement. JAMA : the journal of the American Medical Association. 1999;282(15):1458-65.
247. Glauser TA, Nevins PH, Williamson JC, Abdolrasulnia M, Salinas GD, Zhang J, et al. Adherence to the 2007 cystic fibrosis pulmonary guidelines: a national survey of CF care centers. Pediatric pulmonology. 2012;47(5):434-40.
248. Miller MB, Gilligan PH. Laboratory aspects of management of chronic pulmonary infections in patients with cystic fibrosis. J Clin Microbiol. 2003;41(9):4009-15.
249. Radhakrishnan DK, Corey M, Dell SD. Realities of expectorated sputum collection in the pediatric cystic fibrosis clinic. Archives of pediatrics & adolescent medicine. 2007;161(6):603-6.
250. Wainwright CE, Vidmar S, Armstrong DS, Byrnes CA, Carlin JB, Cheney J, et al. Effect of bronchoalveolar lavage-directed therapy on *Pseudomonas aeruginosa* infection and structural lung injury in children with cystic fibrosis: a randomized trial. JAMA : the journal of the American Medical Association. 2011;306(2):163-71.
251. Williams HD, Davies JC. Basic science for the chest physician: *Pseudomonas aeruginosa* and the cystic fibrosis airway. Thorax. 2012;67(5):465-7.
252. de Vrankrijker AM, Wolfs TF, van der Ent CK. Challenging and emerging pathogens in cystic fibrosis. Paediatric respiratory reviews. 2010;11(4):246-54.
253. Harrison F. Microbial ecology of the cystic fibrosis lung. Microbiology. 2007;153(Pt 4):917-23.
254. Sibley CD, Grinwis ME, Field TR, Eshaghurshan CS, Faria MM, Dowd SE, et al. Culture enriched molecular profiling of the cystic fibrosis airway microbiome. PloS one. 2011;6(7):e22702.

255. VanDevanter DR, LiPuma JJ. Microbial diversity in the cystic fibrosis airways: where is thy sting? Future Microbiology. 2012;7:801-3.
256. Plummer A, Wildman M. Duration of intravenous antibiotic therapy in people with cystic fibrosis. Cochrane Database of Systematic Reviews. 2011(1):CD006682.
257. Dowd SE, Sun Y, Wolcott RD, Domingo A, Carroll JA. Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) for microbiome studies: bacterial diversity in the ileum of newly weaned *Salmonella*-infected pigs. Foodborne Pathogens and Disease. 2008;5(4):459-72.
258. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nature Methods. 2010;7(5):335-6.
259. Haas BJ, Gevers D, Earl AM, Feldgarden M, Ward DV, Giannoukos G, et al. Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. Genome Research. 2011;21(3):494-504.
260. Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 2010;26(19):2460-1.
261. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, et al. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Applied and environmental microbiology. 2006;72(7):5069-72.
262. Rogers GB, Cuthbertson L, Hoffman LR, Wing PA, Pope C, Hooftman DA, et al. Reducing bias in bacterial community analysis of lower respiratory infections. ISME Journal. 2013;7(4):697-706.
263. Ondov BD, Bergman NH, Phillippy AM. Interactive metagenomic visualization in a Web browser. BMC Bioinformatics. 2011;12:385.
264. Matsuda K, Tsuji H, Asahara T, Kado Y, Nomoto K. Sensitive quantitative detection of commensal bacteria by rRNA-targeted reverse transcription-PCR. Applied and Environmental Microbiology. 2007;73(1):32-9.
265. Reid DW, Latham R, Lamont IL, Camara M, Roddam LF. Molecular analysis of changes in *Pseudomonas aeruginosa* load during treatment of a pulmonary exacerbation in cystic fibrosis. Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society. 2013;12(6):688-99.
266. Zhao J, Schloss PD, Kalikin LM, Carmody LA, Foster BK, Petrosino JF, et al. Decade-long bacterial community dynamics in cystic fibrosis airways. Proceedings of

the National Academy of Sciences of the United States of America. 2012;109(15):5809-14.

267. Stressmann FA, Rogers GB, Marsh P, Lilley AK, Daniels TW, Carroll MP, et al. Does bacterial density in cystic fibrosis sputum increase prior to pulmonary exacerbation? Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society. 2011;10(5):357-65.

268. Breidenstein EB, de la Fuente-Nunez C, Hancock RE. *Pseudomonas aeruginosa*: all roads lead to resistance. Trends Microbiol. 2011;19(8):419-26.

269. Fothergill JL, Mowat E, Ledson MJ, Walshaw MJ, Winstanley C. Fluctuations in phenotypes and genotypes within populations of *Pseudomonas aeruginosa* in the cystic fibrosis lung during pulmonary exacerbations. Journal of medical microbiology. 2010;59(Pt 4):472-81.

270. Lipuma JJ. The changing microbial epidemiology in cystic fibrosis. Clinical microbiology reviews. 2010;23(2):299-323.

271. Filkins LM, Hampton TH, Gifford AH, Gross MJ, Hogan DA, Sogin ML, et al. Prevalence of streptococci and increased polymicrobial diversity associated with cystic fibrosis patient stability. Journal of bacteriology. 2012;194(17):4709-17.

272. Rogers GB, Stressmann FA, Koller G, Daniels T, Carroll MP, Bruce KD. Assessing the diagnostic importance of nonviable bacterial cells in respiratory infections. Diagnostic microbiology and infectious disease. 2008;62(2):133-41.

273. Rogers GB, Marsh P, Stressmann AF, Allen CE, Daniels TV, Carroll MP, et al. The exclusion of dead bacterial cells is essential for accurate molecular analysis of clinical samples. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2010;16(11):1656-8.

274. Erb-Downward JR, Thompson DL, Han MK, Freeman CM, McCloskey L, Schmidt LA, et al. Analysis of the lung microbiome in the "healthy" smoker and in COPD. PloS one. 2011;6(2):e16384.

275. Elizur A, Cannon CL, Ferkol TW. Airway inflammation in cystic fibrosis. Chest. 2008;133(2):489-95.

276. Hausler M, Schweizer K, Biesterfel S, Opladen T, Heimann G. Peripheral decrease and pulmonary homing of CD4+CD45RO+ helper memory T cells in cystic fibrosis. Respiratory medicine. 2002;96(2):87-94.

277. Autissier P, Soulas C, Burdo TH, Williams KC. Evaluation of a 12-color flow cytometry panel to study lymphocyte, monocyte, and dendritic cell subsets in humans.

Cytometry Part A : the journal of the International Society for Analytical Cytology. 2010;77(5):410-9.

278. Martin E, Treiner E, Duban L, Guerri L, Laude H, Toly C, et al. Stepwise development of MAIT cells in mouse and human. PLoS biology. 2009;7(3):e54.

279. Hamerman JA, Ogasawara K, Lanier LL. NK cells in innate immunity. Current opinion in immunology. 2005;17(1):29-35.

280. Culley FJ. Natural killer cells in infection and inflammation of the lung. Immunology. 2009;128(2):151-63.

281. Wesselkamper SC, Eppert BL, Motz GT, Lau GW, Hassett DJ, Borchers MT. NKG2D is critical for NK cell activation in host defense against *Pseudomonas aeruginosa* respiratory infection. Journal of immunology. 2008;181(8):5481-9.

282. Daley L, Pier GB, Liporace JD, Eardley DD. Polyclonal B cell stimulation and interleukin 1 induction by the mucoid exopolysaccharide of *Pseudomonas aeruginosa* associated with cystic fibrosis. Journal of immunology. 1985;134(5):3089-93.

283. Novak J, Dobrovolny J, Novakova L, Kozak T. The decrease in number and change in phenotype of mucosal-associated invariant T cells in the elderly and differences in males and females of reproductive age. Scandinavian journal of immunology. 2014.

284. Lopez-Sagaseta J, Dulberger CL, McFedries A, Cushman M, Saghatelian A, Adams EJ. MAIT recognition of a stimulatory bacterial antigen bound to MR1. Journal of immunology. 2013;191(10):5268-77.

285. McAllister F, Henry A, Kreindler JL, Dubin PJ, Ulrich L, Steele C, et al. Role of IL-17A, IL-17F, and the IL-17 receptor in regulating growth-related oncogene-alpha and granulocyte colony-stimulating factor in bronchial epithelium: implications for airway inflammation in cystic fibrosis. Journal of immunology. 2005;175(1):404-12.

286. Grimaldi D, Le Bourhis L, Sauneuf B, Dechartres A, Rousseau C, Ouaaz F, et al. Specific MAIT cell behaviour among innate-like T lymphocytes in critically ill patients with severe infections. Intensive care medicine. 2014;40(2):192-201.

287. Wong EB, Akilimali NA, Govender P, Sullivan ZA, Cosgrove C, Pillay M, et al. Low levels of peripheral CD161++CD8+ mucosal associated invariant T (MAIT) cells are found in HIV and HIV/TB co-infection. PloS one. 2013;8(12):e83474.

288. Jiang J, Wang X, An H, Yang B, Cao Z, Liu Y, et al. Mucosal-associated Invariant T-Cell Function Is Modulated by Programmed Death-1 Signaling in Patients with Active Tuberculosis. American journal of respiratory and critical care medicine. 2014;190(3):329-39.

289. Cosgrove C, Ussher JE, Rauch A, Gartner K, Kurioka A, Huhn MH, et al. Early and nonreversible decrease of CD161++ /MAIT cells in HIV infection. Blood. 2013;121(6):951-61.
290. Leeansyah E, Ganesh A, Quigley MF, Sonnerborg A, Andersson J, Hunt PW, et al. Activation, exhaustion, and persistent decline of the antimicrobial MR1-restricted MAIT-cell population in chronic HIV-1 infection. Blood. 2013;121(7):1124-35.
291. Gold MC, Cerri S, Smyk-Pearson S, Cansler ME, Vogt TM, Delepine J, et al. Human mucosal associated invariant T cells detect bacterially infected cells. PLoS biology. 2010;8(6):e1000407.
292. Shoki AH, Mayer-Hamblett N, Wilcox PG, Sin DD, Quon BS. Systematic review of blood biomarkers in cystic fibrosis pulmonary exacerbations. Chest. 2013;144(5):1659-70.
293. Levy H, Kalish LA, Huntington I, Weller N, Gerard C, Silverman EK, et al. Inflammatory markers of lung disease in adult patients with cystic fibrosis. Pediatric pulmonology. 2007;42(3):256-62.
294. Mott LS, Park J, Murray CP, Gangell CL, de Klerk NH, Robinson PJ, et al. Progression of early structural lung disease in young children with cystic fibrosis assessed using CT. Thorax. 2012;67(6):509-16.
295. Pressler T. Targeting airway inflammation in cystic fibrosis in children: past, present, and future. Paediatric drugs. 2011;13(3):141-7.
296. Kinnula VL, Crapo JD. Superoxide dismutases in the lung and human lung diseases. American journal of respiratory and critical care medicine. 2003;167(12):1600-19.
297. MacNee W. Oxidative stress and lung inflammation in airways disease. European journal of pharmacology. 2001;429(1-3):195-207.
298. Iobbi-Nivol C, Leimkuhler S. Molybdenum enzymes, their maturation and molybdenum cofactor biosynthesis in Escherichia coli. Biochimica et biophysica acta. 2013;1827(8-9):1086-101.
299. Stojanovic D, Nikic D, Lazarevic K. The level of nickel in smoker's blood and urine. Central European journal of public health. 2004;12(4):187-9.
300. Chen LC, Lippmann M. Effects of metals within ambient air particulate matter (PM) on human health. Inhalation toxicology. 2009;21(1):1-31.
301. Pizzichini E, Pizzichini MM, Efthimiadis A, Hargreave FE, Dolovich J. Measurement of inflammatory indices in induced sputum: effects of selection of sputum to

minimize salivary contamination. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 1996;9(6):1174-80.

302. Domej W, Krachler M, Schlagenhaufen C, Trinker M, Krejs GJ, Irogolic KJ. Trace elements in pleural effusions. Journal of trace elements in medicine and biology : organ of the Society for Minerals and Trace Elements. 1997;11(4):232-8.

303. Rodushkin I, Odman F, Branth S. Multielement analysis of whole blood by high resolution inductively coupled plasma mass spectrometry. Fresenius' Journal of Analytical Chemistry. 1999;364(4):338-46.

304. Minoia C, Sabbioni E, Apostoli P, Pietra R, Pozzoli L, Gallorini M, et al. Trace element reference values in tissues from inhabitants of the European community. I. A study of 46 elements in urine, blood and serum of Italian subjects. The Science of the total environment. 1990;95:89-105.

305. Prohaska C, Pomazal K, Steffan I. Determination of Ca, Mg, Fe, Cu, and Zn in blood fractions and whole blood of humans by ICP-OES. Fresenius' journal of analytical chemistry. 2000;367(5):479-84.

306. Goulle JP, Mahieu L, Castermant J, Neveu N, Bonneau L, Laine G, et al. Metal and metalloid multi-elementary ICP-MS validation in whole blood, plasma, urine and hair. Reference values. Forensic science international. 2005;153(1):39-44.

307. Hood MI, Skaar EP. Nutritional immunity: transition metals at the pathogen-host interface. Nature reviews Microbiology. 2012;10(8):525-37.

308. Wark PA, Johnston SL, Moric I, Simpson JL, Hensley MJ, Gibson PG. Neutrophil degranulation and cell lysis is associated with clinical severity in virus-induced asthma. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 2002;19(1):68-75.

309. Pukhalsky AL, Kapranov NI, Kalashnikova EA, Shmarina GV, Shabalova LA, Kokarovtseva SN, et al. Inflammatory markers in cystic fibrosis patients with lung *Pseudomonas aeruginosa* infection. Mediators of inflammation. 1999;8(3):159-67.

310. Kim J, Molina RM, Donaghey TC, Buckett PD, Brain JD, Wessling-Resnick M. Influence of DMT1 and iron status on inflammatory responses in the lung. American journal of physiology Lung cellular and molecular physiology. 2011;300(4):L659-65.

311. Sardesai VM. Molybdenum: an essential trace element. Nutrition in clinical practice : official publication of the American Society for Parenteral and Enteral Nutrition. 1993;8(6):277-81.

312. Yoshida M, Ota S, Fukunaga K, Nishiyama T. Serum molybdenum concentration in healthy Japanese adults determined by inductively coupled plasma-mass spectrometry. Journal of trace elements in medicine and biology : organ of the Society for Minerals and Trace Elements. 2006;20(1):19-23.
313. Schobert M, Jahn D. Anaerobic physiology of *Pseudomonas aeruginos*a in the cystic fibrosis lung. International journal of medical microbiology : IJMM. 2010;300(8):549-56.
314. Noriega C, Hassett DJ, Rowe JJ. The mobA gene is required for assimilatory and respiratory nitrate reduction but not xanthine dehydrogenase activity in *Pseudomonas aeruginosa*. Current microbiology. 2005;51(6):419-24.
315. Bussiere FI, Gueux E, Rock E, Girardeau JP, Tridon A, Mazur A, et al. Increased phagocytosis and production of reactive oxygen species by neutrophils during magnesium deficiency in rats and inhibition by high magnesium concentration. The British journal of nutrition. 2002;87(2):107-13.
316. Mulcahy H, Lewenza S. Magnesium limitation is an environmental trigger of the *Pseudomonas aeruginosa* biofilm lifestyle. PloS one. 2011;6(8):e23307.
317. Johnson MD, Garrett CK, Bond JE, Coggan KA, Wolfgang MC, Redinbo MR. *Pseudomonas aeruginosa* PilY1 binds integrin in an RGD- and calcium-dependent manner. PloS one. 2011;6(12):e29629.
318. Sarkisova S, Patrauchan MA, Berglund D, Nivens DE, Franklin MJ. Calcium-induced virulence factors associated with the extracellular matrix of mucoid *Pseudomonas aeruginosa* biofilms. Journal of bacteriology. 2005;187(13):4327-37.
319. Denning GM, Railsback MA, Rasmussen GT, Cox CD, Britigan BE. Pseudomonas pyocyanine alters calcium signaling in human airway epithelial cells. The American journal of physiology. 1998;274(6 Pt 1):L893-900.
320. Abdulhamid I, Beck FW, Millard S, Chen X, Prasad A. Effect of zinc supplementation on respiratory tract infections in children with cystic fibrosis. Pediatric pulmonology. 2008;43(3):281-7.
321. Holgate ST, Yang Y, Haitchi HM, Powell RM, Holloway JW, Yoshisue H, et al. The genetics of asthma: ADAM33 as an example of a susceptibility gene. Proceedings of the American Thoracic Society. 2006;3(5):440-3.
322. Sagel SD, Chmiel JF, Konstan MW. Sputum biomarkers of inflammation in cystic fibrosis lung disease. Proceedings of the American Thoracic Society. 2007;4(4):406-17.

323. Bartlett JR, Friedman KJ, Ling SC, Pace RG, Bell SC, Bourke B, et al. Genetic modifiers of liver disease in cystic fibrosis. JAMA : the journal of the American Medical Association. 2009;302(10):1076-83.
324. Sun L, Rommens JM, Corvol H, Li W, Li X, Chiang TA, et al. Multiple apical plasma membrane constituents are associated with susceptibility to meconium ileus in individuals with cystic fibrosis. Nature genetics. 2012;44(5):562-9.
325. Agudo A, Bonet C, Sala N, Munoz X, Aranda N, Fonseca-Nunes A, et al. Hemochromatosis (*HFE*) gene mutations and risk of gastric cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC) study. Carcinogenesis. 2013;34(6):1244-50.
326. Gannon PO, Medelci S, Le Page C, Beaulieu M, Provencher DM, Mes-Masson AM, et al. Impact of hemochromatosis gene (*HFE*) mutations on epithelial ovarian cancer risk and prognosis. International journal of cancer. 2011;128(10):2326-34.
327. Gehrke SG, Stremmel W, Mathes I, Riedel HD, Bents K, Kallinowski B. Hemochromatosis and transferrin receptor gene polymorphisms in chronic hepatitis C: impact on iron status, liver injury and HCV genotype. Journal of molecular medicine. 2003;81(12):780-7.
328. Nandar W, Connor JR. *HFE* gene variants affect iron in the brain. The Journal of nutrition. 2011;141(4):729S-39S.
329. Moran A, Becker D, Casella SJ, Gottlieb PA, Kirkman MS, Marshall BC, et al. Epidemiology, pathophysiology, and prognostic implications of cystic fibrosis-related diabetes: a technical review. Diabetes care. 2010;33(12):2677-83.
330. Colombo C, Ellemunter H, Houwen R, Munck A, Taylor C, Wilschanski M, et al. Guidelines for the diagnosis and management of distal intestinal obstruction syndrome in cystic fibrosis patients. Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society. 2011;10 Suppl 2:S24-8.
331. Kerem E, Corey M, Kerem B, Durie P, Tsui LC, Levison H. Clinical and genetic comparisons of patients with cystic fibrosis, with or without meconium ileus. Journal of Pediatrics. 1989;114(5):767-73.
332. Schluchter MD, Konstan MW, Drumm ML, Yankaskas JR, Knowles MR. Classifying severity of cystic fibrosis lung disease using longitudinal pulmonary function data. American journal of respiratory and critical care medicine. 2006;174(7):780-6.

333. Cukjati M, Vaupotic T, Rupreht R, Curin-Serbec V. Prevalence of H63D, S65C and C282Y hereditary hemochromatosis gene mutations in Slovenian population by an improved high-throughput genotyping assay. BMC medical genetics. 2007;8:69.
334. Franklin MJ, Hall S, Taylor PJ. A semi-automated method for the measurement of hepcidin in plasma by on-line extraction coupled to liquid chromatography-tandem mass spectrometry. Clinical Chemistry. 2010;57(Suppl.):A171.
335. Dorfman R, Sandford A, Taylor C, Huang B, Frangolias D, Wang Y, et al. Complex two-gene modulation of lung disease severity in children with cystic fibrosis. The Journal of clinical investigation. 2008;118(3):1040-9.
336. Eisen DP. Mannose-binding lectin deficiency and respiratory tract infection. Journal of innate immunity. 2010;2(2):114-22.
337. Chalmers JD, Fleming GB, Hill AT, Kilpatrick DC. Impact of mannose-binding lectin insufficiency on the course of cystic fibrosis: A review and meta-analysis. Glycobiology. 2011;21(3):271-82.
338. O'May CY, Sanderson K, Roddam LF, Kirov SM, Reid DW. Iron-binding compounds impair *Pseudomonas aeruginosa* biofilm formation, especially under anaerobic conditions. Journal of medical microbiology. 2009;58(Pt 6):765-73.
339. Moreau-Marquis S, O'Toole GA, Stanton BA. Tobramycin and FDA-approved iron chelators eliminate *Pseudomonas aeruginosa* biofilms on cystic fibrosis cells. American journal of respiratory cell and molecular biology. 2009;41(3):305-13.
340. Li Z, Lai HJ, Kosorok MR, Laxova A, Rock MJ, Splaingard ML, et al. Longitudinal pulmonary status of cystic fibrosis children with meconium ileus. Pediatric pulmonology. 2004;38(4):277-84.
341. Gibson G. Rare and common variants: twenty arguments. Nature reviews Genetics. 2011;13(2):135-45.
342. Talmud PJ, Hingorani AD, Cooper JA, Marmot MG, Brunner EJ, Kumari M, et al. Utility of genetic and non-genetic risk factors in prediction of type 2 diabetes: Whitehall II prospective cohort study. British Medical Journal. 2010;340:b4838.
343. Aregbesola A, Voutilainen S, Virtanen JK, Mursu J, Tuomainen TP. Body iron stores and the risk of type 2 diabetes in middle-aged men. European journal of endocrinology / European Federation of Endocrine Societies. 2013;169(2):247-53.
344. Ellervik C, Mandrup-Poulsen T, Tybjaerg-Hansen A, Nordestgaard BG. Total and cause-specific mortality by elevated transferrin saturation and hemochromatosis

genotype in individuals with diabetes: two general population studies. Diabetes care. 2014;37(2):444-52.

345. Moczulski DK, Grzeszczak W, Gawlik B. Role of hemochromatosis C282Y and H63D mutations in *HFE* gene in development of type 2 diabetes and diabetic nephropathy. Diabetes care. 2001;24(7):1187-91.

346. Nelson JE, Brunt EM, Kowdley KV, Nonalcoholic Steatohepatitis Clinical Research N. Lower serum hepcidin and greater parenchymal iron in nonalcoholic fatty liver disease patients with C282Y *HFE* mutations. Hepatology. 2012;56(5):1730-40.

347. Ryan Caballes F, Sendi H, Bonkovsky HL. Hepatitis C, porphyria cutanea tarda and liver iron: an update. Liver international : official journal of the International Association for the Study of the Liver. 2012;32(6):880-93.

348. Ellervik C, Birgens H, Tybjaerg-Hansen A, Nordestgaard BG. Hemochromatosis genotypes and risk of 31 disease endpoints: meta-analyses including 66,000 cases and 226,000 controls. Hepatology. 2007;46(4):1071-80.

349. Boyle MP. Strategies for identifying modifier genes in cystic fibrosis. Proceedings of the American Thoracic Society. 2007;4(1):52-7.

350. Elborn JS, Prescott RJ, Stack BH, Goodchild MC, Bates J, Pantin C, et al. Elective versus symptomatic antibiotic treatment in cystic fibrosis patients with chronic Pseudomonas infection of the lungs. Thorax. 2000;55(5):355-8.

351. Burrows JA, Nissen LM, Kirkpatrick CM, Bell SC. Beta-lactam allergy in adults with cystic fibrosis. Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society. 2007;6(4):297-303.

352. Griffiths AL, Wurzel DF, Robinson PJ, Carzino R, Massie J. Australian epidemic strain pseudomonas (AES-1) declines further in a cohort segregated cystic fibrosis clinic. Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society. 2012;11(1):49-52.

353. Cuthbertson L, Rogers GB, Walker AW, Oliver A, Hoffman LR, Carroll MP, et al. Implications of multiple freeze-thawing on respiratory samples for culture-independent analyses. Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society. 2014.

354. van Bokhoven MA, van Deursen CT, Swinkels DW. Diagnosis and management of hereditary haemochromatosis. British Medical Journal. 2011;342:c7251.

355. Santos PC, Krieger JE, Pereira AC. Molecular diagnostic and pathogenesis of hereditary hemochromatosis. International journal of molecular sciences. 2012;13(2):1497-511.
356. Smith DJ, Anderson GJ, Bell SC, Reid DW. Elevated metal concentrations in the CF airway correlate with cellular injury and disease severity. Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society. 2014;13(3):289-95.
357. Frazier MD, Mamo LB, Ghio AJ, Turi JL. Hepcidin expression in human airway epithelial cells is regulated by interferon-gamma. Respiratory research. 2011;12:100.
358. Nguyen NB, Callaghan KD, Ghio AJ, Haile DJ, Yang F. Hepcidin expression and iron transport in alveolar macrophages. American journal of physiology Lung cellular and molecular physiology. 2006;291(3):L417-25.

Appendix 1. Published works incorporated in the thesis

Targeting iron uptake to control *Pseudomonas aeruginosa* infections in cystic fibrosis

Daniel J. Smith[1,2,3], Iain L. Lamont[4], Greg J. Anderson[3] and David W. Reid[1,2,3]

Affiliations: [1]Dept of Thoracic Medicine, The Prince Charles Hospital, Brisbane, [2]School of Medicine, University of Queensland, Brisbane, and [3]The Iron Metabolism Laboratory, QIMR Berghofer Medical Research Institute, Herston, Australia. [4]Dept of Biochemistry, University of Otago, Dunedin, New Zealand.

Correspondence: D.J. Smith, Dept of Thoracic Medicine, The Prince Charles Hospital, Rode Road, Chermside, Brisbane, Queensland 4032, Australia. E-mail: Daniel_j_smith@health.qld.gov.au

ABSTRACT The aerobic Gram-negative bacterium *Pseudomonas aeruginosa* is an opportunistic pathogen responsible for life-threatening acute and chronic infections in humans. As part of chronic infection *P. aeruginosa* forms biofilms, which shield the encased bacteria from host immune clearance and provide an impermeable and protective barrier against currently available antimicrobial agents.

P. aeruginosa has an absolute requirement for iron for infection success. By influencing cell–cell communication (quorum sensing) and virulence factor expression, iron is a powerful regulator of *P. aeruginosa* behaviour. Consequently, the imposed perturbation of iron acquisition systems has been proposed as a novel therapeutic approach to the treatment of *P. aeruginosa* biofilm infection.

In this review, we explore the influence of iron availability on *P. aeruginosa* infection in the lungs of the people with the autosomal recessive condition cystic fibrosis as an archetypal model of chronic *P. aeruginosa* biofilm infection. Novel therapeutics aimed at disrupting *P. aeruginosa* are discussed, with an emphasis placed on identifying the barriers that need to be overcome in order to translate these promising *in vitro* agents into effective therapies in human pulmonary infections.

@ERSpublications
Can targeting iron uptake be utilised to control *Pseudomonas aeruginosa* infections in cystic fibrosis patients? http://ow.ly/pcLtw

Received: Aug 08 2012 | Accepted after revision: Oct 15 2012 | First published online: Nov 08 2012

Support statement: D.J. Smith is the recipient of a Postgraduate Scholarship from the National Health and Medical Research Council of Australia. G.J. Anderson is the recipient of a Senior Research Fellowship from the National Health and Medical Research Council of Australia. I.L. Lamont is the recipient of funding support from CureKids, New Zealand and the Cystic Fibrosis Association of New Zealand. D.W. Reid is the recipient of a Practitioner Fellowship from the National Health and Medical Research Council of Australia and is also a recipient of a Queensland Health Clinical Fellowship.

Conflict of interest: Disclosures can be found alongside the online version of this article at www.erj.ersjournals.com

Introduction

Pseudomonas aeruginosa is an aerobic Gram-negative bacterium which is widespread in the terrestrial environment. It is extremely robust and capable of surviving in challenging and varied environmental niches, as exemplified by its isolation from jet plane fuel and bottles of disinfectant fluid [1]. This adaptability is conferred by its large genome (approximately 6 Mb) and ability to survive as either a planktonic organism or as a member of a codependent bacterial community within the confines of a "biofilm" [2, 3].

The genetic plasticity and biofilm-forming attributes of *P. aeruginosa* make it a highly successful pathogen in multiple disease settings in eukaryotes. In humans, *P. aeruginosa* is an opportunistic pathogen, which is responsible for life-threatening acute infections in burn victims and other critically ill patients, as well as chronic infections and acute exacerbations in patients with respiratory diseases [4–6].

Iron is essential to the survival of virtually all prokaryotes and eukaryotes. The importance of iron to *P. aeruginosa* is exemplified by the fact that 6% of its transcribed genes are iron-responsive [7]. The concentration of bioavailable iron is a powerful regulator of *P. aeruginosa* behaviour, influencing intercellular communication and biofilm formation [7].

In this review we explore how iron availability within the lung influences the development of chronic *P. aeruginosa* biofilm infection in people with the autosomal recessive genetic disorder cystic fibrosis (CF), and examine current research into how the iron dependency of *P. aeruginosa* may be targeted therapeutically.

The susceptibility of the CF airway to infection

The CF airway is inherently prone to infection. In health, the luminal surface of the respiratory epithelium is coated with airway surface liquid (ASL), comprised of mucins, immune cells and antimicrobial peptides. ASL traps and kills inhaled pathogens which are then rapidly cleared by the mucociliary escalator. In CF, impaired function of the CF transmembrane conductance regulator (CFTR) on respiratory epithelial cells results in increased reabsorption of water from the airway lumen and dehydration of the ASL, with consequent slowing of mucociliary clearance [8]. In addition, defective CFTR-mediated bicarbonate export has been shown in animal models to result in a fall in ASL pH and further inhibition of ASL antimicrobial activity [9]. A similar acidic environment exists in human disease [10]. These alterations in the biophysical properties of ASL are compounded by deficits in airway innate immune defences, including defective iron sequestration and degradation of antimicrobial peptides by high concentrations of endogenous and bacterial-derived proteases, which produce an environment conducive to chronic infection [11, 12].

As CF lung disease progresses, plugging of distal airways by dehydrated, inspissated mucus creates microaerobic or frankly anaerobic pockets within the normally aerobic environment [13, 14]. This low oxygen environment drives phenotypic adaptation in incumbent bacteria and promotes the survival of bacteria capable of existing at low oxygen tensions [13]. Bacterial respiration may further lower oxygen tensions and potentially contribute to alteration in the pH of ASL, which will further impair the bactericidal effects of several antibiotics (especially aminoglycosides) commonly used in CF [13, 15].

Respiratory tract infections in CF begin very early in life [16]. Initial intermittent infections are typically caused by the common respiratory pathogens *Staphylococcus aureus* and nontypeable *Haemophilus influenzae* [17]. By adulthood, a chronic polymicrobial airway infection develops, with *P. aeruginosa* becoming the dominant pathogen in 80% of cases [17, 18]. Chronic *P. aeruginosa* infection leads to an increased rate of lung function decline, morbidity and mortality [19]. Recent culture-independent (metagenomic) microbiological techniques suggest that a wide range of additional bacterial species may also infect the CF airway (including anaerobes), although little is currently known about the pathological significance of these microbes [20, 21]. A key factor in the interplay between host tissues and bacterial pathogens is the management of iron metabolism. The lung is exposed daily to a high oxygen concentration, and unbound iron in atmospheric particulate matter can potentially catalyse the formation of reactive oxygen species, as can ferrous and ferric iron in ASL. This provides the lung with unique challenges with regards to iron homeostasis [22]. Airway cells rapidly sequester iron to prevent the generation of damaging free radicals, and to withhold this key nutrient from inhaled pathogens. This is achieved through uptake of nonprotein-bound iron by divalent metal-ion transporter 1 on the apical surface of bronchial epithelial cells and by the secretion of the iron chelating proteins lactoferrin and transferrin into ASL [23].

The lung is highly adept at iron detoxification and iron is barely detectable in normal airway secretions. The resulting lack of accessible iron inhibits the growth of infectious bacteria. However, respiratory secretions and sputum from patients with CF contain micromolar concentrations of iron, making this micronutrient more readily available to inhaled pathogens (airway iron indices from existing studies are presented in

DOI: 10.1183/09031936.00124012

TABLE 1 Studies reporting iron concentration in respiratory secretions from patients with cystic fibrosis (CF)

First author [ref.]	Year	Population	Controls	Substrate	Assay	Iron concentration	
						Controls	CF
Gifford [27]	2011	Adults	None	Expectorated sputum	Inductively coupled plasma mass spectrometry	Not available	Stable 1.11 (0.09–4.01) mg·mL^{-1} Exacerbation 2.22 (0.77–7.04) mg·mL^{-1}
Reid [28]	2007	Adult and paediatric	Healthy	Expectorated sputum	Colorimetric	0 (0–15.8) µmol·L^{-1}	*Pseudomonas aeruginosa* infection 34 (2.4–78) µmol·L^{-1} No *P. aeruginosa* infection 18 (8–118) µmol·L^{-1}
Reid [25]	2004	Adult	Healthy	Expectorated sputum	Colorimetric	0 (0–13.2) µmol·L^{-1}	Stable 33.3 (0–111.2) µmol·L^{-1} Exacerbation 44.4 (17.0–128.7) µmol·L^{-1}
Stites [24]	1999	Adult	Healthy nonsmokers	BALF	Colorimetric	0±0 µg·dL^{-1}	42±11.6 µg·dL^{-1}
Stites [26]	1998	Adult	Nonsmokers recent URTI	Expectorated sputum	Coulometry	0±0 ng·mg^{-1}	242±47 ng·mg^{-1}

Data are presented as median (range) or mean±SD, unless otherwise stated. BALF: bronchoalveolar lavage fluid; URTI: upper respiratory tract infection.

DOI: 10.1183/09031936.00124012

table 1) [24–28]. *In vitro* data suggest that this increase in lung iron may partly be due to defective iron handling by CF bronchial epithelial (CFBE) cells [12].

Neutrophils represent the first line of cellular defence against bacterial pathogens and also participate in iron-withholding by secreting lactoferrin and lipocalins. Lipocalin 2 binds and inactivates bacterial-derived iron scavenging molecules (siderophores), although it is not thought to bind to the *P. aeruginosa*-derived siderophores [29, 30]. The role of lipocalin 2 in the setting of polymicrobial infection has not been explored in CF, although serum levels increase when patients develop an increased infective burden [31].

The development of *P. aeruginosa* biofilms in CF airways

Following initial airway infection, planktonic *P. aeruginosa* undergoes rapid phenotypic and genotypic adaptation to prevent immune recognition. This is achieved by the formation of a biofilm, which offers physical protection and downregulation of virulence factors [2, 32].

Biofilms comprise an extracellular matrix (ECM) of exopolysaccharides, extracellular DNA (eDNA) and proteins produced by the resident bacteria. By trapping essential nutrients and providing a physical barrier to host immune attack, biofilms offer a survival advantage to embedded bacteria. In the CF lung it is proposed that *P. aeruginosa* binds abnormal mucins present in ASL to form biofilm "rafts" which float on the respiratory epithelium [32]. Established biofilm infections cannot be eradicated with currently available antibiotics or by the host's neutrophilic inflammatory response [33].

Biofilm development is largely determined by its environment and available nutrients [34]. *In vitro*, biofilms develop complex three-dimensional structures containing phenotypically distinct subpopulations of bacteria connected by water channels formed within the ECM [35]. Iron is essential as a bacterial nutrient, and lack of iron interferes with biofilm development [36]. Iron also contributes to the structural integrity of the biofilm by cross-linking exopolysaccharide strands [37].

P. aeruginosa biofilm development is dependent on cell–cell communication. Quorum sensing is a population density dependent form of communication employed by bacteria to control the synthesis of key regulatory proteins. Quorum sensing is integral to all activities of the bacterial community, including biofilm formation. *P. aeruginosa* employs three quorum sensing systems (Las, Rhl and *Pseudomonas* quinolone signal (PQS)), each of which is iron responsive [38–41].

Under conditions of limited iron availability, both the Las and Rhl systems are activated [38, 39]. The relationship between the PQS system and iron is complex. PQS is able to operate as an iron chelator, thereby controlling the activation of the Las and Rhl systems through iron limitation [42]. Conversely, PQS synthesis is increased under conditions of both iron limitation and excess [40].

An important gene cluster under the control of Rhl is the *rhlAB* operon that regulates production of the biosurfactant rhamnolipid. Rhamnolipid acts as a "wetting agent", reducing surface tension and promoting surface-associated movement (twitching motility). Correctly timed production of small quantities of rhamnolipid is critical for the production of water channels within the core of mature *P. aeruginosa* biofilms, through which motile bacteria are able to travel. *In vitro*, inhibition of rhamnolipid production leads to the formation of flat, thick, immature biofilms [43]. In contrast, excessive rhamnolipid promotes the dispersal of mature biofilms and causes newly formed biofilms to be thin and flat [44, 45]. When iron availability is limited, increased rhamnolipid production and twitching motility prevents biofilm development, or triggers biofilm dispersal, depending on the stage of biofilm maturation [44, 46].

Paradoxically, supraphysiological iron concentrations appear to be detrimental to biofilm development. Normal human plasma contains 20–25 μM of iron, <1 μM of which is protein-bound. Biofilms grown in medium containing 100 μM of iron contain less eDNA, fail to develop complex macrocolonies and are more susceptible to antimicrobials compared with biofilms grown in an equivalent 1 μM iron medium [47]. Similarly, exposing established biofilms to medium containing 200 μM of ferric ammonium citrate triggers dispersal events and facilitates antibiotic killing [48].

Iron acquisition by *P. aeruginosa*

P. aeruginosa may take up iron from either haem or nonhaem iron sources (fig. 1). Two haem uptake systems have been described in *P. aeruginosa* (Phu and Has) [49]. The Phu system relies on direct binding of haem or haem-containing proteins to a membrane-bound receptor, whereas the Has system secretes a haem-binding protein (HasAp) which is reabsorbed through the Has receptor (HasR) when bound to haem [50, 51]. The *P. aeruginosa* genome contains a third haem receptor-encoding gene (*hxuC*); however, its functional regulation has yet to be characterised [52]. It is unknown whether haem uptake systems are employed by *P. aeruginosa* within the CF lung; however, patients frequently have frank blood in their sputum and subclinical bleeding into the airway is probably common.

DOI: 10.1183/09031936.00124012

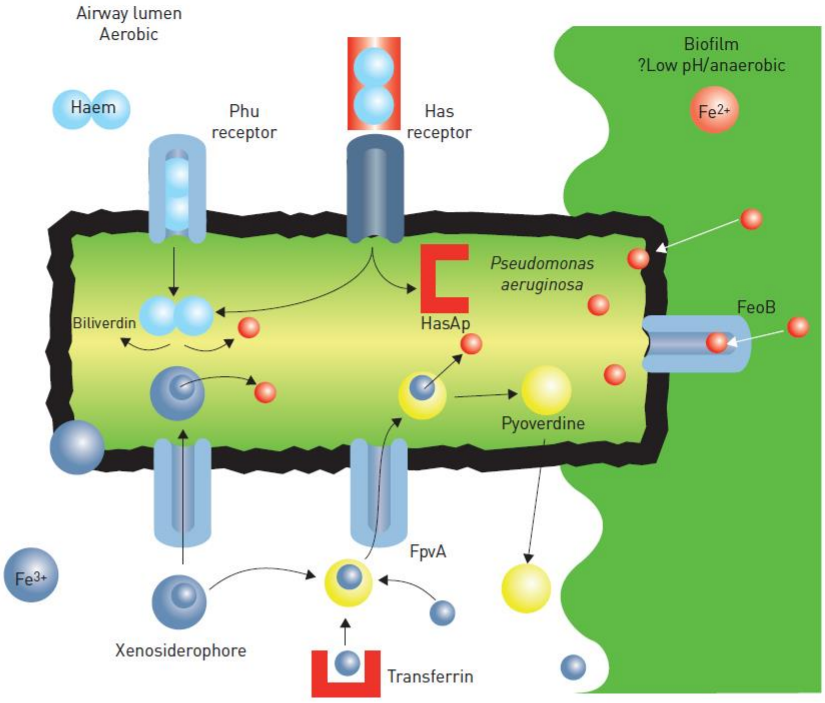


FIGURE 1 *Pseudomonas aeruginosa* iron acquisition pathways.

Haem is an uncommon iron source in the natural environment and *P. aeruginosa* must also be capable of scavenging nonhaem iron, which under aerobic conditions is most probably present in the poorly soluble ferric (Fe^{3+}) form. *P. aeruginosa* (and other bacteria and fungi) therefore produces high-affinity iron chelating siderophores [53]. Siderophores are secreted by *P. aeruginosa* into the local environment to chelate free iron and "strip" iron from host iron-binding proteins.

Two distinct siderophores have been characterised in *P. aeruginosa*: pyoverdine and pyochelin. >50 distinct pyoverdine subtypes have been characterised and are responsible for the distinctive yellow-green fluorescence of certain pseudomonads [54]. Pyoverdines are the primary siderophore produced by *P. aeruginosa*, with one of three distinct subclasses being produced by individual strains [55].

Pyochelin is considered a secondary siderophore in *P. aeruginosa*, having a much lower iron binding affinity than pyoverdine [52, 53]. Pyochelin appears to have less influence on the biofilm forming capacity of *P. aeruginosa* than pyoverdine, and its importance for iron acquisition during clinical airway infections is unclear [36, 53]. In addition to acquiring iron using autologous siderophores, *P. aeruginosa* has a high capacity to take up iron-laden siderophores produced by other bacteria and fungi [52].

P. aeruginosa, while naturally an aerobic bacterium, is capable of adapting to low oxygen environments such as those encountered within plugged CF airways. Within these regions of low oxygen tension and low pH there is potential for the redox status of iron to change to the more "soluble" ferrous (Fe^{2+}) form, but there are currently no data on this scenario in CF lung disease. Ferrous iron may be acquired by *P. aeruginosa* by passive diffusion or uptake through the FeoB receptor, although the role of these mechanisms in the clinical setting is at present unclear [56].

P. aeruginosa iron acquisition systems are tightly controlled by the ferric uptake regulator (Fur). Fur acts both directly and indirectly, through extracytoplasmic sigma factors (including PvdS), to limit iron absorption [57]. Under iron-replete conditions, Fur binds ferrous iron and attaches to a consensus sequence (Fur-box) in the promoter region of genes instrumental in iron acquisition, thus suppressing their transcription [58]. In the presence of iron, Fur inhibits iron conservation strategies by suppressing the production of two small RNAs (PrrF1 and PrrF2) [59]. In the absence of iron these small RNAs are

DOI: 10.1183/09031936.00124012

synthesised and facilitate inhibition of genes that encode "nonessential" iron-containing proteins, thereby maintaining the cytoplasmic iron pool for essential use [60]. Under low iron environments siderophore synthesis increases and nonessential iron-consuming processes are downregulated. Several excellent comprehensive reviews of the iron acquisition systems employed by *P. aeruginosa* have recently been published [36, 52, 53, 57, 60], but the above overview highlights the central role of iron in *P. aeruginosa* biofilm development.

Targeting bacterial iron acquisition as a therapeutic strategy

The critical role of iron in *P. aeruginosa* survival and biofilm formation may represent a potential "Achilles' heel" in the defensive armamentarium of this fastidious pathogen. Thus considerable research endeavours on a variety of fronts are being undertaken to develop novel therapeutic strategies based on disruption of bacterial iron homeostasis. These therapeutic strategies may be particularly important in CF where host iron homeostatic mechanisms appear to be abnormal.

Delivering toxic amounts of iron to P. aeruginosa

In vitro studies have suggested that iron-laden synthetic chelators can be utilised to deliver high concentrations of iron to biofilm-dwelling *P. aeruginosa* with resultant biofilm disruption [61]. While this approach demonstrates promise *in vitro*, the high redox activity of iron and potential for harmful reactive oxygen species generation within the human airway must be considered. Animal studies suggest that iron loading can potentiate proinflammatory cytokine responses to *P. aeruginosa* lipopolysaccharide and increase lung injury, highlighting the potential danger of iron therapy [62]. Furthermore, detrimental effects of iron in the lung are well described [63], and this may potentially be accentuated in the CF lung where iron handling appears to be defective [12].

Iron mimetics

Gallium (Ga^{3+}) has a similar ionic radius to Fe^{3+} and is mistaken for Fe^{3+} by many biological systems. However, Ga^{3+} lacks the redox activity of iron and consequently competitively inhibits iron-dependent processes [64]. *In vitro* studies have shown that Ga^{3+} can prevent the growth of planktonic and biofilm-dwelling *P. aeruginosa* and disperse established biofilms, with transcriptomic analysis suggesting that this effect is mediated through inhibition of iron acquisition systems including repression of *pvdS* gene [65]. Mouse infection models have demonstrated "cure" of *P. aeruginosa*-induced pneumonia and wound infections by local application of Ga^{3+} [65, 66]. A preparation of gallium conjugated to the siderophore desferrioxamine is undergoing *in vitro* and animal studies. This preparation aims to utilise the siderophore to improve delivery of gallium to biofilm-dwelling bacteria. Initial studies indicate that this agent has powerful anti-*P. aeruginosa* biofilm actions, in particular when combined with the aminoglycoside antibiotic gentamicin [67].

Gallium salts have established medical applications in the systemic treatment of malignant hypercalcaemia and in the diagnostic imaging of haematological malignancies [68]. Currently licensed preparations have poor oral bioavailability and are associated with a risk of nephrotoxicity, diarrhoea, hypocalcaemia, microcytic anaemia and immunosuppression when administered systemically [68]. Although the risk of toxicity is acceptably low when Ga^{3+} is used in short courses for currently licensed indications, little is known about its cumulative toxicity when used in long-term maintenance regimens as would probably be required to prevent *P. aeruginosa* infection in the CF airway. A safety study of intravenous gallium nitrate (Ganite; Genta Inc., Berkeley Heights, NJ, USA) (dose regimen 100 or 200 $mg \cdot m^{-2} \cdot day^{-1}$ for 5 days) in patients with CF was commenced in April 2010 and the results of this study are awaited (clinicaltrials.gov/ct2/show/NCT01093521).

An inhalational preparation of gallium would potentially overcome the obstacle of poor bioavailability and deliver high concentrations to biofilms while limiting systemic toxicity, but there are limited data about the safety of this approach. Gallium arsenide is utilised in the microelectronics industry and has undergone toxicological studies to assess the risk to workers from inhalation exposure [68]. Reported changes induced by gallium arsenide inhalation or tracheal instillation in animal models include epithelial hyperplasia, squamous metaplasia, benign and malignant lung tumours, and haematological malignancy [68]. Although these side-effects may be attributed to arsenide, a potentially toxic effect of gallium must also be considered. To the best of our knowledge the safety of gallium nitrate by inhalation in animal models has only been reported in abstract form [69]. In this single study, no excess toxicity was demonstrated; however, dosing was limited to a single 6-h exposure.

DOI: 10.1183/09031936.00124012

Iron chelators

Exogenously administered, high-affinity iron chelators may be utilised to out-compete *P. aeruginosa* siderophores for available iron. Two such approaches have been proposed, first through the use of naturally occurring biological chelators such as lactoferrin, and secondly through the administration of entirely synthetic compounds.

Biological iron chelators

Lactoferrin

Lactoferrin is an antimicrobial glycoprotein with iron chelating properties. Lactoferrin represents a major endogenous antimicrobial constituent of airway secretions [70]. In addition to iron chelation, lactoferrin may induce bacterial cell lysis through interactions with lipopolysaccarhide and it may also prevent bacterial invasion of epithelial cells through competitive binding and proteolytic degradation of surface associated adhesion proteins [71].

In the presence of intense neutrophilic inflammation, as seen in CF airway infection, lactoferrin concentrations would be expected to be greatly elevated in respiratory secretions. However, the CF lung displays relatively low levels of lactoferrin, which are most depleted in the presence of *P. aeruginosa* [72]. This reduction is due partly to proteolytic degradation by high concentrations of proteases present in the CF airways, which serves to increase susceptibility to *P. aeruginosa* infection and promote biofilm growth [72].

In vitro, lactoferrin is capable of inhibiting *P. aeruginosa* biofilm development; however, there is conflicting evidence over whether or not this is mediated through iron chelation [73–75]. In pivotal studies conducted by SINGH [75] and others, lactoferrin induced twitching motility and repressed biofilm formation in a manner similar to that seen with iron limitation. Similarly, the biofilm-disrupting effects of apo-lactoferrin were neutralised by pre-loading lactoferrin with iron, suggesting that at least some of the effect was mediated by iron chelation [74, 75]. However, O'MAY *et al.* [73] demonstrated that the efficacy of lactoferrin in biofilm disruption was augmented at higher iron concentrations (250–500 μM), suggesting an iron chelation-independent method of biofilm disruption.

The efficacy of lactoferrin supplementation *in vivo* is beginning to be investigated. However, the potential for proteolytic degradation may impact on the clinical efficacy of this therapeutic approach *in vivo*.

Lactoferrin combined with hypothiocyanate

Production of hypothiocyanate in ASL is another important innate immune defence strategy that appears to be defective in CF lung [11]. Hypothiocyanate is normally formed by the oxidation of thiocyanate, but CF epithelial cells do not secrete thiocyanate [11]. A combination preparation of lactoferrin and hypothiocyanate (Meveol; Alaxia, Lyon, France) delivered by inhalation is undergoing development (www.alaxia-pharma.eu/meveol), and has been granted orphan drug status to promote clinical trials. To date, *in vitro* and animal data demonstrating its antimicrobial actions have only been presented in abstract form.

Synthetic iron chelators

Synthetic iron chelators developed primarily for the treatment of conditions associated with systemic iron overload display much higher iron binding affinities than biological iron-carrying proteins and therefore potentially offer greater competition to bacterial siderophores. A number of authors have reported on the ability of these agents to disrupt *P. aeruginosa* biofilms; however, the bacterial strains studied, chelators employed and culture models utilised have varied between studies (table 2).

MOREAU-MARQUIS *et al.* [76] investigated the effects of the currently licensed iron chelators deferasirox and deferoxamine on *P. aeruginosa* biofilms grown on CF epithelial cells. These studies indicated that both agents were able to prevent biofilm growth as well as disrupt established biofilms. Their efficacy was further enhanced when they were co-administered with the antipseudomonal antibiotic tobramycin.

In addition to demonstrating the antibiofilm properties of a number of synthetic chelators, O'MAY *et al.* [73] showed an increased efficacy of these agents against anaerobically grown biofilms, highlighting the important role that local environmental conditions may play when these interventions are deployed *in vivo*. In similar experiments, BANIN *et al.* [30] demonstrated disruption of *P. aeruginosa* PAO1 biofilms by EDTA, which was augmented by the aminoglycoside gentamicin. However, in contrast, LIU *et al.* [77] suggested that EDTA administered alone could potentiate PAO1 biofilm formation, yet it inhibited biofilm growth when co-administered with the efflux pump inhibitor phenyl-arginine-β-naphthylamide. Possible explanations for the different findings in these two studies include differences in biofilm model, the ability of EDTA to chelate multiple divalent cations in addition to Fe^{2+} and the chelator concentrations used (14.6 $\mu g \cdot mL^{-1}$ *versus* 5 $\mu g \cdot mL^{-1}$) [30, 77].

DOI: 10.1183/09031936.00124012

TABLE 2 *In vitro* studies employing synthetic iron chelators in the treatment of *Pseudomonas aeruginosa* biofilms

First author [ref.]	Year	Iron chelators	Adjuvant treatment	Biofilm model employed	Outcomes
Liu [77]	2010	2DP Acetohydroxamic acid EDTA	PaβN	Coverslip	EDTA, 2DP and acetohydroxamic acid each worked synergistically to reduce biofilm growth EDTA alone increased biofilm growth
Moreau-Marquis [76]	2009	Deferasirox Deferoxamine	Tobramycin	CFBE cell-lined flow cells Static CFBE cells Abiotic static culture	Deferasirox and deferoxamine reduced biofilm growth on CFBE cells and potentiated the effects of tobramycin Deferasirox and deferoxamine acted synergistically with tobramycin to disrupt biofilms grown on CFBE cells Neither deferasirox nor deferoxamine disrupted biofilms on abiotic static surfaces
O'May [73]	2009	DPTA Deferoxamine 2DP EDDA EDTA		Borosilicate glass tubes (aerobic and anaerobic) Flow cells	2DP, DPTA and EDTA impaired biofilm growth 2DP disrupted established biofilms Antibiofilm effects of all iron chelators were greatest against anaerobically cultured biofilms
Banin [30]	2006	EDTA	Gentamicin	Disk reactor Flow cells	EDTA reduced biofilm-associated cells by >99% EDTA increased biofilm dispersal events Coadministration of gentamicin increased bacterial killing Antibiofilm effects were overcome by divalent cationic Mg^{2+}, Ca^{2+} and Fe^{2+}

2DP: 2,2,dipyridyl; PaβN: phenyl-arginine-β-naphthylamide; CFBE: cystic fibrosis bronchial epithelial; DTPA: diethylenetriaminepentacetic acid; EDDA: ethylenediamine-N,N9-diacetic acid.

Siderophore–antibiotic conjugates and the "Trojan horse" approach

Reduced membrane permeability, antibiotic efflux pumps and antimicrobial inactivating enzymes (*e.g.* β-lactamases) are defence strategies employed by biofilm-dwelling bacteria which augment the physical protection offered by the ECM. The essential requirement for iron trafficking mediated by siderophores in biofilm-dwelling pseudomonads has driven the concept of "hijacking" this system to circumvent the protection offered by the ECM and cell membrane impermeability. As a result, siderophore–antibiotic conjugates (SACs) have been developed which may function as "Trojan horses" [78–80].

Naturally occurring SACs termed sideromycins were discovered many years prior to the description of siderophore trafficking [80]. Sideromycins are produced by *Actinomyces* and *Streptomyces* species as antimicrobials against competing micro-organisms. These agents rely heavily upon their recognition by the iron uptake system of the target species and, disappointingly, they display limited activity against *P. aeruginosa* [81, 82].

Penicillin–siderophore conjugates have been proposed as leading candidates for synthetic SACs. These compounds have the advantage of having a distinct antibiotic active site and siderophore conjugation site, which means that there is no need for the antibiotic to dissociate from the siderophore to exert its effect. Furthermore, the antibacterial action of penicillin is exerted through attachment to penicillin binding proteins located in the periplasm. Thus, the conjugated molecule needs only traverse the bacterial outer membrane to be effective. Recent *in vitro* and mouse model data have demonstrated that an ampicillin-based SAC has superior antibacterial actions against a range of laboratory and clinical strains of *P. aeruginosa* (and other Gram-negative bacteria) compared to the commonly prescribed antipseudomonal antibiotics meropenem, imipenem and ciprofloxacin [83]. Similar *in vitro* experiments performed with β-lactam antibiotics conjugates have yielded mixed results [78, 84]. A sulfactam-containing SAC has demonstrated potent activity against multi-antibiotic resistant *P. aeruginosa* strains (minimum inhibitory concentration required to produce 90% inhibition 8 $\mu g \cdot mL^{-1}$), whereas a monobactam SAC demonstrated only modest improvements in minimum inhibitory concentrations against "epidemic" CF *P. aeruginosa* strains when compared to established antipseudomonal antibiotics [78, 84].

DOI: 10.1183/09031936.00124012

Other potential targets based on iron homeostasis

Additional potential strategies to disrupt *P. aeruginosa* iron homeostasis include competitive inhibition of siderophore uptake through the use of siderophore mimetics or monoclonal antibodies, which bind to bacterial siderophore receptors but do not deliver bioavailable iron [85, 86]. These techniques are in their infancy and there is little published work on the effect of these strategies with regards to *P. aeruginosa*. Such therapies are likely to be very expensive.

Advances in crystallography are defining the structural composition of enzymes involved in bacterial siderophore synthesis, which may lead to targeted inhibitors of these pathways. Characterisation of the structure of salicylation enzymes involved in the synthesis of siderophores by *Mycobacterium tuberculosis* and *Yersinia pestis* have resulted in the development of the synthetic compound 5-O-(N-salicylsulfamoyl)adenosine (salicyl-AMS), which has been shown to inhibit the growth of both *M. tuberculosis* and *Y. pestis* under iron-limiting conditions [87]. The design of similar agents that are active against *P. aeruginosa* has yet to be described, although they are likely to be developed in time.

Finally, iron acquisition pathways may be targeted in vaccine development. Attempts to develop clinically efficacious vaccines against *P. aeruginosa* have, to date, been unsuccessful [88]. Obstacles include *P. aeruginosa*'s multiple antigenic determinants, multiple serotypes of these determinants between clinical strains and the different expression of determinants under different conditions (*e.g.* planktonic and biofilm growth) [89]. Application of proteomic and bioinformatics techniques to the study of uropathogenic *Escherichia coli* identified six highly conserved iron uptake surface membrane receptors [90]. Deployment of a polyvalent vaccine against three of these receptors in a murine model resulted in effective protection against urinary tract infection [90]. *P. aeruginosa* iron-regulated outer membrane proteins are also immunogenic, but their potential as vaccine targets has not been explored [91].

Strategies to limit iron in the setting of a polymicrobial infection

Any new intervention directed against *P. aeruginosa* must consider the potential impact on copathogens, as suppression of the dominant pathogen may allow the emergence of other, potentially more harmful, infections.

In common with *P. aeruginosa*, other commonly isolated CF airway pathogens, including *S. aureus*, *H. influenzae* and *Burkholderia cepacia* complex (BCC), are capable of biofilm development and each have an absolute requirement for iron [92–96].

In a single published study on the effect of gallium on planktonic and biofilm grown BCC, strains were exposed to gallium nitrate at concentrations of up to 64 $mg \cdot L^{-1}$ (~250 μM Ga^{3+}) [97]. Disappointingly, there was little effect seen on either planktonic or biofilm growth. These results have been challenged on the basis that the concentration of gallium used was lower than could be safely administered therapeutically [98]. However, in a similar study examining the effects of gallium maltolate on the growth of *S. aureus* and *S. epidermidis* biofilms, equally disappointing results were reported, and minimal inhibitory concentrations far in excess of those that could be safely administered systemically (>3000 $mg \cdot L^{-1}$) were needed to achieve biofilm inhibition [99].

There are few studies of iron chelator effects on CF bacterial pathogens other than *P. aeruginosa* (table 3) [100–103]. The effect of the synthetic chelators deferiprone and deferoxamine against a number of staphylococcal species grown in broth cultures has been examined [103]. Deferiprone inhibited growth of all species studied, but desferrioxamine promoted growth in a number of staphylococcal species [103]. Similarly, it has been demonstrated that *S. aureus* can take up iron hydroxamates such as desferrioxamine and utilise them as an iron source to promote biofilm growth [101, 104].

Translational research and the challenges of targeting *P. aeruginosa* iron homeostasis in the human lung

Despite the early promise of a number of the agents discussed above *in vitro*, important questions remain to be answered about their safety and efficacy before advancing to human trials.

The majority of the work presented above has been performed using common laboratory-adapted strains of *P. aeruginosa*, which vary both genetically and phenotypically from clinical strains isolated from the CF lung. Additionally, studies have considered only a limited number of environmental variables and often use conditions that are distinct from those within the CF lung, where there is reduced oxygen tension, significant amounts of extracellular iron, low pH and a hostile milieu replete with proteases and free radicals [2, 10, 13]. In the very limited work performed with clinical isolates, different responses to iron-targeted therapies have been reported, both between clinical and laboratory strains, and between clinical isolates from different patients [73].

DOI: 10.1183/09031936.00124012

TABLE 3 The effect of iron chelators on common cystic fibrosis airway pathogens

First author [ref.]	Year	Bacteria tested	Iron chelators	Culture model employed	Outcomes
Aguila [102]	2001	*S. aureus* (clinical and laboratory isolates); MRSA	Lactoferrin	Broth cultures in iron depleted minimal media or normal human serum	Lactoferrin was bacteriostatic against most clinical and laboratory strains, including many antibiotic-resistant strains
Percival [100]	2005	*S. aureus*; *S. epidermidis*; *P. aeruginosa*; MRSA; *E. coli*; *K. pneumoniae*	EDTA	Silicon tubing, central venous catheter model	Exposure of catheter-related biofilm to EDTA for 25 h inhibited biofilm growth of all bacterial species
Kim [103]	2009	*S. aureus*; *S. epidermidis*; *S. saprophyticus*	DFO Deferiprone	Broth culture in minimal media	DFO promoted the growth of some species (especially *S. aureus*) Deferiprone inihibited the growth of all species tested
Al-Azemi [101]	2011	*S. aureus*[#]	EDTA DFO	Coverslip static biofilm	EDTA impaired biofilm growth DFO at low concentration (100 μM) stimulated biofilm growth DFO concentrations >1 mM inhibited growth EDTA and DFO displayed synergistic antibiofilm effects

S. aureus: *Staphylococcus aureus*; MRSA: methicillin-resistant *S. aureus*; *S. epidermidis*: *Staphylococcus epidermidis*; *P. aeruginosa*: *Pseudomonas aeruginosa*; *E. coli*: *Escherichia coli*; *K. pneumoniae*: *Klebsiella pneumoniae*; *S. saprophyticus*: *Staphylococcus saprophyticus*; DFO: desferrioxamine B. [#]: Panton–Valentine leukocidin-positive community-acquired methicillin-sensitive *S. aureus*.

Although there have been no studies of treatments targeting bacterial iron homeostasis under "CF lung conditions", factors including pH, glucose source and oxygen availability have been shown to affect the biofilm-forming capacity of airway pathogens [32, 73, 101]. Consequently, if new agents are to be successful they must remain active over a wide pH range, and compete with both ferrous and ferric iron acquisition systems.

Iron limitation *in vitro* triggers the dispersal of motile planktonic bacteria with increased virulence compared to their biofilm-dwelling counterparts, and thus the potential for biofilm disruption to trigger an acute host inflammatory response [105]. To better understand the inflammatory potential of these agents testing in an animal model is desirable; however, representative models of CF airway infection are limited. Mice containing the major *CFTR* gene mutations (*e.g.* DeltaF508, G551D) do not develop spontaneous airway infections and *P. aeruginosa* has to be introduced directly into the mouse lung where it is either spontaneously cleared or results in overwhelming infection [106, 107]. Successful chronic mouse airway infection has been achieved by introducing *P. aeruginosa* bound to agar beads into the trachea and by contaminating drinking water with *P. aeruginosa* [108], but how closely this reflects human disease is debated. More recently, pig and ferret models of CF have been developed, which may more closely mimic human respiratory disease [109, 110].

Finally, the route of administration must be considered. The concentrations of gallium required for activity against *S. aureus* and BCC biofilms are well above those considered safe for systemic delivery in humans, suggesting that inhalation may be the only viable option to safely administer the required dose. Similarly, *in vitro* studies suggest iron chelators delivered directly to biofilms grown on the apical membrane of CFBE cells inhibit growth more effectively than when they are applied to the basal membrane, suggesting that direct delivery to the airway may also be the preferred mode of delivery for these compounds [76]. The possibility of localised delivery of chelators is supported by *in vitro* modelling, which has suggested that chelated iron may be effectively aerosolised to a particle size suitable for lung delivery [61].

Conclusions

As our understanding of the biology of bacterial biofilms expands, new therapeutic possibilities present themselves. Given the absolute requirement for iron of *P. aeruginosa* and other CF airway pathogens, disrupting iron utilisation is an exciting avenue for further research. The results of the safety trial of intravenous gallium are eagerly awaited. Future studies of iron chelation therapy will need to test the efficacy of these agents against clinically relevant *P. aeruginosa* strains and establish their safety within animal models, before proceeding to human trials.

DOI: 10.1183/09031936.00124012

References

1 Kobayashi H, Kobayashi O, Kawai S. Pathogenesis and clinical manifestations of chronic colonization by *Pseudomonas aeruginosa* and its biofilms in the airway tract. *J Infect Chemother* 2009; 15: 125–142.
2 Rau MH, Hansen SK, Johansen HK, *et al*. Early adaptive developments of *Pseudomonas aeruginosa* after the transition from life in the environment to persistent colonization in the airways of human cystic fibrosis hosts. *Environ Microbiol* 2010; 12: 1643–1658.
3 Stover CK, Pham XQ, Erwin AL, *et al*. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* 2000; 406: 959–964.
4 Trautmann M, Lepper PM, Haller M. Ecology of *Pseudomonas aeruginosa* in the intensive care unit and the evolving role of water outlets as a reservoir of the organism. *Am J Infect Control* 2005; 33: S41–S49.
5 Estahbanati HK, Kashani PP, Ghanaatpisheh F. Frequency of *Pseudomonas aeruginosa* serotypes in burn wound infections and their resistance to antibiotics. *Burns* 2002; 28: 340–348.
6 Murphy TF. *Pseudomonas aeruginosa* in adults with chronic obstructive pulmonary disease. *Curr Opin Pulm Med* 2009; 15: 138–142.
7 Vasil ML, Ochsner UA. The response of *Pseudomonas aeruginosa* to iron: genetics, biochemistry and virulence. *Mol Microbiol* 1999; 34: 399–413.
8 Matsui H, Grubb BR, Tarran R, *et al*. Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease. *Cell* 1998; 95: 1005–1015.
9 Pezzulo AA, Tang XX, Hoegger MJ, *et al*. Reduced airway surface pH impairs bacterial killing in the porcine cystic fibrosis lung. *Nature* 2012; 487: 109–113.
10 Tate S, MacGregor G, Davis M, *et al*. Airways in cystic fibrosis are acidified: detection by exhaled breath condensate. *Thorax* 2002; 57: 926–929.
11 Moskwa P, Lorentzen D, Excoffon KJ, *et al*. A novel host defense system of airways is defective in cystic fibrosis. *Am J Respir Crit Care Med* 2007; 175: 174–183.
12 Moreau-Marquis S, Bomberger JM, Anderson GG, *et al*. The ΔF508-CFTR mutation results in increased biofilm formation by *Pseudomonas aeruginosa* by increasing iron availability. *Am J Physiol Lung Cell Mol Physiol* 2008; 295: L25–L37.
13 Worlitzsch D, Tarran R, Ulrich M, *et al*. Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. *J Clin Invest* 2002; 109: 317–325.
14 Sanderson K, Wescombe L, Kirov SM, *et al*. Bacterial cyanogenesis occurs in the cystic fibrosis lung. *Eur Respir J* 2008; 32: 329–333.
15 King P, Citron DM, Griffith DC, *et al*. Effect of oxygen limitation on the *in vitro* activity of levofloxacin and other antibiotics administered by the aerosol route against *Pseudomonas aeruginosa* from cystic fibrosis patients. *Diagn Microbiol Infect Dis* 2010; 66: 181–186.
16 Ranganathan SC, Parsons F, Gangell C, *et al*. Evolution of pulmonary inflammation and nutritional status in infants and young children with cystic fibrosis. *Thorax* 2011; 66: 408–413.
17 Cystic Fibrosis Foundation. Patient Registry 2010: Annual Data Report. Bethesda, USA, 2012.
18 Tunney MM, Field TR, Moriarty TF, *et al*. Detection of anaerobic bacteria in high numbers in sputum from patients with cystic fibrosis. *Am J Respir Crit Care Med* 2008; 177: 995–1001.
19 Emerson J, Rosenfeld M, McNamara S, *et al*. *Pseudomonas aeruginosa* and other predictors of mortality and morbidity in young children with cystic fibrosis. *Pediatr Pulmonol* 2002; 34: 91–100.
20 Jones AM. Anaerobic bacteria in cystic fibrosis: pathogens or harmless commensals? *Thorax* 2011; 66: 558–559.
21 Tunney MM, Klem ER, Fodor AA, *et al*. Use of culture and molecular analysis to determine the effect of antibiotic treatment on microbial community diversity and abundance during exacerbation in patients with cystic fibrosis. *Thorax* 2011; 66: 579–584.
22 Valavanidis A, Fiotakis K, Vlachogianni T. Airborne particulate matter and human health: toxicological assessment and importance of size and composition of particles for oxidative damage and carcinogenic mechanisms. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev* 2008; 26: 339–362.
23 Cole AM, Waring AJ. The role of defensins in lung biology and therapy. *Am J Respir Med* 2002; 1: 249–259.
24 Stites SW, Plautz MW, Bailey K, *et al*. Increased concentrations of iron and isoferritins in the lower respiratory tract of patients with stable cystic fibrosis. *Am J Respir Crit Care Med* 1999; 160: 796–801.
25 Reid DW, Lam QT, Schneider H, *et al*. Airway iron and iron-regulatory cytokines in cystic fibrosis. *Eur Respir J* 2004; 24: 286–291.
26 Stites SW, Walters B, O'Brien-Ladner AR, *et al*. Increased iron and ferritin content of sputum from patients with cystic fibrosis or chronic bronchitis. *Chest* 1998; 114: 814–819.
27 Gifford AH, Miller SD, Jackson BP, *et al*. Iron and CF-related anemia: expanding clinical and biochemical relationships. *Pediatr Pulmonol* 2011; 46: 160–165.
28 Reid DW, Carroll V, O'May C, *et al*. Increased airway iron as a potential factor in the persistence of *Pseudomonas aeruginosa* infection in cystic fibrosis. *Eur Respir J* 2007; 30: 286–292.
29 Flo TH, Smith KD, Sato S, *et al*. Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. *Nature* 2004; 432: 917–921.
30 Banin E, Brady KM, Greenberg EP. Chelator-induced dispersal and killing of *Pseudomonas aeruginosa* cells in a biofilm. *Appl Environ Microbiol* 2006; 72: 2064–2069.
31 Eichler I, Nilsson M, Rath R, *et al*. Human neutrophil lipocalin, a highly specific marker for acute exacerbation in cystic fibrosis. *Eur Respir J* 1999; 14: 1145–1149.
32 Hassett DJ, Korfhagen TR, Irvin RT, *et al*. *Pseudomonas aeruginosa* biofilm infections in cystic fibrosis: insights into pathogenic processes and treatment strategies. *Expert Opin Ther Targets* 2010; 14: 117–130.
33 Bjarnsholt T, Jensen PO, Fiandaca MJ, *et al*. *Pseudomonas aeruginosa* biofilms in the respiratory tract of cystic fibrosis patients. *Pediatr Pulmonol* 2009; 44: 547–558.
34 Klausen M, Heydorn A, Ragas P, *et al*. Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. *Mol Microbiol* 2003; 48: 1511–1124.
35 Klausen M, Aaes-Jorgensen A, Molin S, *et al*. Involvement of bacterial migration in the development of complex multicellular structures in *Pseudomonas aeruginosa* biofilms. *Mol Microbiol* 2003; 50: 61–68.

DOI: 10.1183/09031936.00124012

36 Banin E, Vasil ML, Greenberg EP. Iron and *Pseudomonas aeruginosa* biofilm formation. *Proc Natl Acad Sci USA* 2005; 102: 11076–11081.
37 Chen X, Stewart PS. Role of electrostatic interactions in cohesion of bacterial biofilms. *Appl Microbiol Biotechnol* 2002; 59: 718–720.
38 Bollinger N, Hassett DJ, Iglewski BH, *et al.* Gene expression in *Pseudomonas aeruginosa*: evidence of iron override effects on quorum sensing and biofilm-specific gene regulation. *J Bacteriol* 2001; 183: 1990–1996.
39 Duan K, Surette MG. Environmental regulation of *Pseudomonas aeruginosa* PAO1 Las and Rhl quorum-sensing systems. *J Bacteriol* 2007; 189: 4827–4836.
40 Oglesby AG, Farrow JM 3rd, Lee JH, *et al.* The influence of iron on *Pseudomonas aeruginosa* physiology: a regulatory link between iron and quorum sensing. *J Biol Chem* 2008; 283: 15558–15567.
41 Diggle SP, Matthijs S, Wright VJ, *et al.* The *Pseudomonas aeruginosa* 4-quinolone signal molecules HHQ and PQS play multifunctional roles in quorum sensing and iron entrapment. *Chem Biol* 2007; 14: 87–96.
42 Bredenbruch F, Geffers R, Nimtz M, *et al.* The *Pseudomonas aeruginosa* quinolone signal (PQS) has an iron-chelating activity. *Environ Microbiol* 2006; 8: 1318–1329.
43 Davey ME, Caiazza NC, O'Toole GA. Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* 2003; 185: 1027–1036.
44 Glick R, Gilmour C, Tremblay J, *et al.* Increase in rhamnolipid synthesis under iron-limiting conditions influences surface motility and biofilm formation in *Pseudomonas aeruginosa*. *J Bacteriol* 2010; 192: 2973–2980.
45 Schooling S, Charaf UK, Allison DG, Gilbert P. A role for rhamnolipid in biofilm dispersion. *Biofilms* 2004; 1: 90–99.
46 Patriquin GM, Banin E, Gilmour C, *et al.* Influence of quorum sensing and iron on twitching motility and biofilm formation in *Pseudomonas aeruginosa*. *J Bacteriol* 2008; 190: 662–671.
47 Yang L, Barken KB, Skindersoe ME, *et al.* Effects of iron on DNA release and biofilm development by *Pseudomonas aeruginosa*. *Microbiology* 2007; 153: 1318–1328.
48 Musk DJ, Banko DA, Hergenrother PJ. Iron salts perturb biofilm formation and disrupt existing biofilms of *Pseudomonas aeruginosa*. *Chem Biol* 2005; 12: 789–796.
49 Ochsner UA, Johnson Z, Vasil ML. Genetics and regulation of two distinct haem-uptake systems, Phu and Has, in *Pseudomonas aeruginosa*. *Microbiology* 2000; 146: 185–198.
50 Letoffe S, Redeker V, Wandersman C. Isolation and characterization of an extracellular haem-binding protein from *Pseudomonas aeruginosa* that shares function and sequence similarities with the *Serratia marcescens* HasA haemophore. *Mol Microbiol* 1998; 28: 1223–1234.
51 Alontaga AY, Rodriguez JC, Schonbrunn E, *et al.* Structural characterization of the hemophore HasAp from *Pseudomonas aeruginosa*: NMR spectroscopy reveals protein-protein interactions between Holo-HasAp and hemoglobin. *Biochemistry* 2009; 48: 96–109.
52 Cornelis P. Iron uptake and metabolism in pseudomonads. *Appl Microbiol Biotechnol* 2010; 86: 1637–1645.
53 Lamont IL, Konings AF, Reid DW. Iron acquisition by *Pseudomonas aeruginosa* in the lungs of patients with cystic fibrosis. *Biometals* 2009; 22: 53–60.
54 Visca P, Imperi F, Lamont IL. Pyoverdine siderophores: from biogenesis to biosignificance. *Trends Microbiol* 2007; 15: 22–30.
55 Meyer JM, Stintzi A, De Vos D, *et al.* Use of siderophores to type pseudomonads: the three *Pseudomonas aeruginosa* pyoverdine systems. *Microbiology* 1997; 143: 35–43.
56 Marshall B, Stintzi A, Gilmour C, *et al.* Citrate-mediated iron uptake in *Pseudomonas aeruginosa*: involvement of the citrate-inducible FecA receptor and the FeoB ferrous iron transporter. *Microbiology* 2009; 155: 305–315.
57 Cornelis P, Matthijs S, Van Oeffelen L. Iron uptake regulation in *Pseudomonas aeruginosa*. *Biometals* 2009; 22: 15–22.
58 Prince RW, Cox CD, Vasil ML. Coordinate regulation of siderophore and exotoxin A production: molecular cloning and sequencing of the *Pseudomonas aeruginosa fur* gene. *J Bacteriol* 1993; 175: 2589–2598.
59 Wilderman PJ, Sowa NA, FitzGerald DJ, *et al.* Identification of tandem duplicate regulatory small RNAs in *Pseudomonas aeruginosa* involved in iron homeostasis. *Proc Natl Acad Sci USA* 2004; 101: 9792–9797.
60 Vasil ML. How we learnt about iron acquisition in *Pseudomonas aeruginosa*: a series of very fortunate events. *Biometals* 2007; 20: 587–601.
61 Musk DJ Jr, Hergenrother PJ. Chelated iron sources are inhibitors of *Pseudomonas aeruginosa* biofilms and distribute efficiently in an *in vitro* model of drug delivery to the human lung. *J Appl Microbiol* 2008; 105: 380–388.
62 Le BV, Khorsi-Cauet H, Bach V, *et al.* Modulation of *Pseudomonas aeruginosa* lipopolysaccharide-induced lung inflammation by chronic iron overload in rat. *FEMS Immunol Med Microbiol* 2012; 64: 255–264.
63 Ghio AJ. Disruption of iron homeostasis and lung disease. *Biochim Biophys Acta* 2009; 1790: 731–739.
64 Chitambar CR, Narasimhan J. Targeting iron-dependent DNA synthesis with gallium and transferrin-gallium. *Pathobiology* 1991; 59: 3–10.
65 Kaneko Y, Thoendel M, Olakanmi O, *et al.* The transition metal gallium disrupts *Pseudomonas aeruginosa* iron metabolism and has antimicrobial and antibiofilm activity. *J Clin Invest* 2007; 117: 877–888.
66 DeLeon K, Balldin F, Watters C, *et al.* Gallium maltolate treatment eradicates *Pseudomonas aeruginosa* infection in thermally injured mice. *Antimicrob Agents Chemother* 2009; 53: 1331–1337.
67 Banin E, Lozinski A, Brady KM, *et al.* The potential of desferrioxamine-gallium as an anti-*Pseudomonas* therapeutic agent. *Proc Natl Acad Sci USA* 2008; 105: 16761–16766.
68 Chitambar CR. Medical applications and toxicities of gallium compounds. *Int J Environ Res Public Health* 2010; 7: 2337–2361.
69 Adamcakova-Dodd A, Magwood J, Kim J, *et al.* Toxicity assessment of gallium nitrate inhalation using murine model. American Thoracic Society Annual Scientific Meeting. May 19, 2010, New Orleans. *Am J Respir Crit Care Med* 2010; 181: A6266.
70 Ganz T. Antimicrobial polypeptides in host defense of the respiratory tract. *J Clin Invest* 2002; 109: 693–697.
71 Valenti P, Berlutti F, Conte MP, *et al.* Lactoferrin functions: current status and perspectives. *J Clin Gastroenter* 2004; 38: S127–S129.
72 Rogan MP, Taggart CC, Greene CM, *et al.* Loss of microbicidal activity and increased formation of biofilm due to decreased lactoferrin activity in patients with cystic fibrosis. *J Infect Dis* 2004; 190: 1245–1253.

 DOI: 10.1183/09031936.00124012

73 O'May CY, Sanderson K, Roddam LF, *et al.* Iron-binding compounds impair *Pseudomonas aeruginosa* biofilm formation, especially under anaerobic conditions. *J Med Microbiol* 2009; 58: 765–773.
74 Ammons MC, Ward LS, Dowd S, *et al.* Combined treatment of *Pseudomonas aeruginosa* biofilm with lactoferrin and xylitol inhibits the ability of bacteria to respond to damage resulting from lactoferrin iron chelation. *Int J Antimicrob Agents* 2011; 37: 316–323.
75 Singh PK. Iron sequestration by human lactoferrin stimulates *P. aeruginosa* surface motility and blocks biofilm formation. *Biometals* 2004; 17: 267–270.
76 Moreau-Marquis S, O'Toole GA, Stanton BA. Tobramycin and FDA-approved iron chelators eliminate *Pseudomonas aeruginosa* biofilms on cystic fibrosis cells. *Am J Respir Cell Mol Biol* 2009; 41: 305–313.
77 Liu Y, Yang L, Molin S. Synergistic activities of an efflux pump inhibitor and iron chelators against *Pseudomonas aeruginosa* growth and biofilm formation. *Antimicrob Agents Chemother* 2010; 54: 3960–3963.
78 Livermore DM, Mushtaq S, Warner M. Activity of BAL30376 (monobactam BAL19764 + BAL29880 + clavulanate) *versus* Gram-negative bacteria with characterized resistance mechanisms. *J Antimicrob Chemother* 2010; 65: 2382–2395.
79 Braun V, Braun M. Active transport of iron and siderophore antibiotics. *Curr Opin Microbiol* 2002; 5: 194–201.
80 Budzikiewicz H. Siderophore-antibiotic conjugates used as Trojan horses against *Pseudomonas aeruginosa*. *Curr Top Med Chem* 2001; 1: 73–82.
81 Pramanik A, Stroeher UH, Krejci J, *et al.* Albomycin is an effective antibiotic, as exemplified with *Yersinia enterocolitica* and *Streptococcus pneumoniae*. *Int J Med Microbiol* 2007; 297: 459–469.
82 Braun V, Pramanik A, Gwinner T, *et al.* Sideromycins: tools and antibiotics. *Biometals* 2009; 22: 3–13.
83 Mollmann U, Heinisch L, Bauernfeind A, *et al.* Siderophores as drug delivery agents: application of the "Trojan horse" strategy. *Biometals* 2009; 22: 615–624.
84 Page MG, Dantier C, Desarbre E. *In vitro* properties of BAL30072, a novel siderophore sulfactam with activity against multiresistant Gram-negative bacilli. *Antimicrob Agents Chemother* 2010; 54: 2291–2302.
85 Miethke M, Marahiel MA. Siderophore-based iron acquisition and pathogen control. *Microbiol Mol Biol Rev* 2007; 71: 413–451.
86 Wade WF, O'Toole GA. Antibodies and immune effectors: shaping Gram-negative bacterial phenotypes. *Trends Microbiol* 2010; 18: 234–239.
87 Ferreras JA, Ryu JS, Di Lello F, *et al.* Small-molecule inhibition of siderophore biosynthesis in *Mycobacterium tuberculosis* and *Yersinia pestis*. *Nature Chem Biol* 2005; 1: 29–32.
88 Johansen HK, Gotzsche PC. Vaccines for preventing infection with *Pseudomonas aeruginosa* in cystic fibrosis. *Cochrane Database Syst Rev* 2008; 4: CD001399.
89 Kamei A, Coutinho-Sledge YS, Goldberg JB, *et al.* Mucosal vaccination with a multivalent, live-attenuated vaccine induces multifactorial immunity against *Pseudomonas aeruginosa* acute lung infection. *Infect Immun* 2011; 79: 1289–1299.
90 Alteri CJ, Hagan EC, Sivick KE, *et al.* Mucosal immunization with iron receptor antigens protects against urinary tract infection. *PLoS Pathogens* 2009; 5: e1000586.
91 Shand GH, Pedersen SS, Brown MR, *et al.* Serum antibodies to *Pseudomonas aeruginosa* outer-membrane proteins and iron-regulated membrane proteins at different stages of chronic cystic fibrosis lung infection. *J Med Microbiol* 1991; 34: 203–212.
92 Coenye T. Social interactions in the *Burkholderia cepacia* complex: biofilms and quorum sensing. *Future Microbiol* 2010; 5: 1087–1099.
93 Starner TD, Zhang N, Kim G, *et al. Haemophilus influenzae* forms biofilms on airway epithelia: implications in cystic fibrosis. *Am J Respir Crit Care Med* 2006; 174: 213–220.
94 Yang L, Liu Y, Markussen T, *et al.* Pattern differentiation in co-culture biofilms formed by *Staphylococcus aureus* and *Pseudomonas aeruginosa*. *FEMS Immunol Med Microbiol* 2011; 62: 339–347.
95 Hammer ND, Skaar EP. Molecular mechanisms of *Staphylococcus aureus* iron acquisition. *Ann Rev Microbiol* 2011; 65: 129–147.
96 Morton DJ, Turman EJ, Hensley PD, *et al.* Identification of a siderophore utilization locus in nontypeable *Haemophilus influenzae*. *BMC Microbiol* 2010; 10: 113.
97 Peeters E, Nelis HJ, Coenye T. Resistance of planktonic and biofilm-grown *Burkholderia cepacia* complex isolates to the transition metal gallium. *J Antimicrob Chemother* 2008; 61: 1062–1065.
98 Lechuga-Ballesteros D, Truong-Le V, Patzer EJ. Comment on: Resistance of planktonic and biofilm-grown *Burkholderia cepacia* complex isolates to the transition metal gallium. *J Antimicrob Chemother* 2009; 63: 1078–1079.
99 Baldoni D, Steinhuber A, Zimmerli W, *et al. In vitro* activity of gallium maltolate against Staphylococci in logarithmic, stationary, and biofilm growth phases: comparison of conventional and calorimetric susceptibility testing methods. *Antimicrob Agents Chemother* 2010; 54: 157–163.
100 Percival SL, Kite P, Eastwood K, *et al.* Tetrasodium EDTA as a novel central venous catheter lock solution against biofilm. *Infect Control Hospital Epidemiol* 2005; 26: 515–519.
101 Al-Azemi A, Fielder MD, Abuknesha RA, *et al.* Effects of chelating agent and environmental stresses on microbial biofilms: relevance to clinical microbiology. *J Appl Microbiol* 2011; 110: 1307–1313.
102 Aguila A, Herrera AG, Morrison D, *et al.* Bacteriostatic activity of human lactoferrin against *Staphylococcus aureus* is a function of its iron-binding properties and is not influenced by antibiotic resistance. *FEMS Immunol Med Microbiol* 2001; 31: 145–152.
103 Kim CM, Shin SH. Effect of iron-chelator deferiprone on the *in vitro* growth of staphylococci. *J Korean Med Sci* 2009; 24: 289–295.
104 Sebulsky MT, Heinrichs DE. Identification and characterization of *fhuD1* and *fhuD2*, two genes involved in iron-hydroxamate uptake in *Staphylococcus aureus*. *J Bacteriol* 2001; 183: 4994–5000.
105 VanDevanter DR, Van Dalfsen JM. How much do *Pseudomonas* biofilms contribute to symptoms of pulmonary exacerbation in cystic fibrosis? *Pediatr Pulmonol* 2005; 39: 504–506.
106 Guilbault C, Saeed Z, Downey GP, *et al.* Cystic fibrosis mouse models. *Am J Respir Cell Mol Biol* 2007; 36: 1–7.
107 Wilke M, Buijs-Offerman RM, Aarbiou J, *et al.* Mouse models of cystic fibrosis: phenotypic analysis and research applications. *J Cyst Fibros* 2011; 10: Suppl. 2, S152–S171.

DOI: 10.1183/09031936.00124012

108 Coleman FT, Mueschenborn S, Meluleni G, *et al*. Hypersusceptibility of cystic fibrosis mice to chronic *Pseudomonas aeruginosa* oropharyngeal colonization and lung infection. *Proc Natl Acad Sci USA* 2003; 100: 1949–1954.
109 Stoltz DA, Meyerholz DK, Pezzulo AA, *et al*. Cystic fibrosis pigs develop lung disease and exhibit defective bacterial eradication at birth. *Sci Transl Med* 2010; 2: 29ra31.
110 Sun X, Sui H, Fisher JT, *et al*. Disease phenotype of a ferret CFTR-knockout model of cystic fibrosis. *J Clin Invest* 2010; 120: 3149–3160.

DOI: 10.1183/09031936.00124012

REVIEW

Treatment of pulmonary exacerbations in cystic fibrosis

People with the genetic condition cystic fibrosis suffer repeated respiratory tract infections from an early age, which are often superseded by chronic airway infection, with resultant progressive loss of lung function and premature death from respiratory failure. Acute pulmonary exacerbations contribute to diminished quality of life, reduced lung function, morbidity and mortality in cystic fibrosis, and remain a major obstacle to improving long term outcomes. This article discusses current diagnostic and therapeutic strategies employed in the treatment of pulmonary exacerbations, with a focus on critical appraisal of the evidence based on which strategies are founded. Areas of uncertainty are highlighted and current and potential future research directions are discussed.

KEYWORDS: antibiotics · cystic fibrosis · definition · etiology · infection · treatment

Daniel J Smith[†1,2,3], David W Reid[1,2,3] & Scott C Bell[1,2,4]

[1]Department of Thoracic Medicine, The Prince Charles Hospital, Rode Road, Chermside, Brisbane, Queensland, 4032, Australia
[2]School of Medicine, The University of Queensland, The Prince Charles Hospital, Queensland, Chermside, Brisbane, Queensland, 4032, Australia
[3]Queensland Institute of Medical Research, Brisbane, Queensland, 4006, Australia
[4]Queensland Children's Medical Research Institute, Level 4 Foundation Building, Royal Children's Hospital, 300 Herston Road, Herston, 4029, Queensland, Australia
[†]Author for correspondence:
Tel.: +61 731 394 770
Fax: +61 731 395 630
daniel_j_smith@health.qld.gov.au

Advances in cystic fibrosis (CF) care, including better nutrition, aggressive treatment of infection, and the development of specialist center care, have led to substantial improvements in life expectancy [1]. However, the majority of patients still die from complications associated with chronic respiratory sepsis before their fifth decade of life. Equally, improvements in survival have come at the cost of treatment-related morbidity including the emergence of multidrug resistant infections, complications of indwelling intravenous lines, and allergic and toxic effects of antibiotics [2–4]. Awareness of the potential pitfalls of treatment places the onus on the treating CF team to ensure that therapies are used in a way that maximizes their effect, while minimizing exposure to harm.

Pulmonary exacerbations are characterized by increasing respiratory symptoms and acute deterioration of lung function. They are associated with significant morbidity and mortality and may accelerate lung function decline [5,6], as well as being associated with diminished quality of life [7] and increased healthcare costs [8].

The availability of high-quality clinical trials to guide therapy in CF is limited and this is particularly true of exacerbations. Similarly, there have been multiple studies into airway inflammation and bacterial infection during exacerbations, but no therapies have been developed based on new knowledge generated. Thus, treatment decisions are often based on clinical experience and anecdote. Barriers to study design include inadequate cohort sizes to adequately powered single center trials, limited pharmaceutical industry investment in multicenter studies, and differences in study designs required to satisfy drug licensing authorities internationally.

The aim of this article is to critically appraise the current evidence base on which strategies for treatment of pulmonary exacerbations are founded, and discuss how treatment is commonly implemented where evidence is lacking. We highlight current areas of uncertainty, and propose areas in which further research is required.

Defining an exacerbation

In 1994 the Cystic Fibrosis Foundation (USA) convened a consensus conference to define outcome measures for use in CF clinical trials [9]. One of the eight 'top priority' issues identified was the development of a standard definition of a pulmonary exacerbation that could be applied consistently within clinical trials. Despite this welcome initiative, 17 years later no universally accepted definition has been realized. The development of an exacerbation definition is confounded by the natural day-to-day variability of symptoms, poor correlation between biomarkers and clinical outcomes [10], and differences in hospital admission and antibiotic administration policies between CF centers and across healthcare settings [11].

The absence of a universally accepted definition of what constitutes an exacerbation makes direct comparison between rates and severity of pulmonary exacerbations reported in clinical trials difficult. Other important uses of a definition include the ability to track changes in disease

future medicine part of fsg

epidemiology, and their utility as decision aids with respect to determining when treatment of an exacerbation can be safely discontinued.

In attempting to formulate a standard definition of pulmonary exacerbations, several authors have adopted the approach of performing component analysis of previous studies to examine for the factors most likely to prompt the treating physician to make the diagnosis of an exacerbation [12–14]. In these studies, symptoms generally outperformed physical examination findings and investigations. Increased cough, and increased sputum volume or purulence, and decreased appetite and weight were found to be most predictive (TABLE 1). The findings of these studies have not been incorporated into a unifying definition and instead, multiple different and invalidated scoring systems remain the norm [15,16].

A fall in lung function is usually seen during a pulmonary exacerbation, therefore incorporating spirometric measures into the definition of a pulmonary exacerbation may be reasonable. However, in up to a quarter of patients lung function fails to return to baseline by the end of treatment suggesting its use in guiding treatment duration may be limited [17].

Given the hypothesis that exacerbations are precipitated by an imbalance between host immunity and infection, research has, and continues to focus on identifying biomarkers capable of detecting exacerbation onset and recovery. The ideal biomarker of exacerbations should maintain its sensitivity with varying disease severity, be minimally impacted on by independent factors (e.g., age and sex), and respond rapidly with clinical improvement and resolution of inflammation [10].

Table 1. Symptom profiles used in previous attempts to define a pulmonary exacerbation.

Signs and symptoms (new or increased)	Fuchs	Ramsey	ARIC	RSSQ
Pulmonary signs and symptoms				
Increased dyspnoea with exertion	X			X
Decreased exercise tolerance				X
Increased work of breath			X	
Cough	X	X		X
Day cough			X	
Night cough			X	
Wet or congested cough			X	
Chest congestion	X			X
Frequency of cough	X			
Cough up mucus	X			
Wheezing			X	
Hemoptysis/coughing up blood	X		X	X
Sputum volume	X	X	X	X
Change in sputum appearance		X		X
Change in sputum colour			X	X
Change in sputum consistency			X	X
Increased respiratory rate		X		
Decreased lung function	X	X		
Upper respiratory tract symptoms				
Sore throat/runny nose		X	X	
Sinus pain/tenderness	X			X
Change in sinus discharge	X			X
Constitutional and GI signs and symptoms				
Malaise/fatigue/lethargy	X			X
Abdominal pain				
Fever	X	X		X
Decreased appetite/anorexia	X	X		X
Weight loss		X		X
Work/school absenteeism		X		X

ARIC: Acute Respiratory Illness Checklist; RSSQ: Respiratory and Systemic Questionnaire (© BoehringerIngelheim). Reproduced with permission from [42].

Levels of the acute phase reactant, C-reactive protein (CRP), increase in the presence of systemic inflammation, and in particular in the setting of bacterial infections. In CF, CRP levels often increase at the onset of an exacerbation, and decrease with the administration of antibiotics [18,19]. However CRP levels have been shown to return to normal before an improvement in either clinical status or lung function is seen, suggesting it has limited utility as a guide to discontinuation of therapy [20]. Furthermore, changes in CRP with treatment vary widely between individuals, and have even been shown to increase in some patients despite clinical response [20].

Airway inflammation in CF is neutrophil predominant [21]. The chemokine IL-8 is a major neutrophil chemoattractant, and can be detected in high levels in CF sputum [18]. Studies have demonstrated a fall in sputum IL-8 levels in response to antibiotic therapy, and a correlation with improvement in lung function [18]. However, results have been inconsistent, both between studies and between individuals within studies [10,19,22]. This variability may reflect differing sampling techniques (induced vs expectorated sputum) and the response of IL-8 may depend on the degree of disease severity (i.e., less reduction in IL-8 in the setting of more advanced lung disease). Another confounding factor is the anatomical heterogeneity in lung disease and difference in sputum purulence depending on sample origin within the lung. Similar difficulties have been found with a range of other pro- and anti-inflammatory cytokines (including IL-6, IL-10, TNF-α) and neutrophil products (including neutrophils elastase complexes and myeloperoxidase) that have been assessed as biomarkers [10,22].

Neutrophil elastase (NE) is a proteolytic enzyme released by activated neutrophils that is believed to contribute to host tissue damage at sites of inflammation. Neutrophils derived from patients with CF spontaneously release increased amounts of NE, and NE can be detected in high concentrations in airway secretions [10,23]. A number of studies have reported a fall in NE in response to treatment of an exacerbation, but again results between studies have not been consistent [23,24]. Calprotectin is also produced by activated neutrophils in response to pro-inflammatory cytokines and has been validated as a fecal marker of inflammatory bowel disease [25], but it is also detectable in CF serum and airway secretions. A recent study has demonstrated decreases in sputum and serum calprotectin levels with treatment of pulmonary exacerbations and changes in sputum calprotectin demonstrated a stronger correlation with clinical improvement when compared with IL-8, VEGF or myeloperoxidase [19]. Future studies will delineate whether this marker will prove to be more specific than its predecessors.

Exhaled breath condensates (EBC) measure volatile substances that are produced in the lungs and appear in expired air. They have potential advantages over sputum sampling by providing a summated assessment of the lung, and can be measured in both young children, and patients unable to produce expectorated sputum samples. The reported differences in EBC from CF patients when compared with healthy controls include lower pH, nitric oxide and ammonia concentrations, and increased concentrations of IL-6, IL-8, leukotriene B4 and nitrites [26–30]. However, as with sputum biomarkers, reported changes during exacerbations and their treatment have been inconsistent [27–29].

An alternate approach to measuring the inflammatory response is to measure changes in airway microbial flora. At the most basic level this can be achieved by determining sputum bacterial concentrations before and after treatment. When this has been done most studies report a fall in bacterial load accompanying clinical improvement [31]. However, airway infection in CF is polymicrobial [32], and it is still not known which organisms are the most important during exacerbations, and consequently which should be monitored to indicate response [33].

In the setting of chronic bacterial infection, the growth of subpopulations of bacteria with increased virulence may also be important. Detection of either these subpopulations or the virulence factors that they produce may facilitate diagnosis of exacerbations. *Pseudomonas aeruginosa* (the major pathogen of chronic CF airways infection) is capable of rapid changes in both genotype and phenotype during exacerbations [34]. Potential indicators of *P. aeruginosa* virulence detectable in CF sputum and EBC include pyocyanin, quorum sensing (QS) signals, exotoxins and pyoverdine, as well as the appearance of *P. aeruginosa* small colony variants [35–38]. There are currently only limited clinical data to support the use of virulence factors to monitor response. Grimwood *et al.* demonstrated an increased concentration of *P. aeruginosa* exo-enzymes in CF patients' sputum during acute exacerbations compared with stable controls, and that concentrations fell in response to antibiotics [39]. In a more recent study, Fothergill

et al. examined a panel of *P. aeruginosa* virulence factors and described differing patterns of response to antibiotics in each of the three patients studied [34].

Future perspective

Future research will need to focus on longitudinal studies correlating biomarkers with changes in lung function, symptoms and antibiotic response. It is unlikely that a single biomarker will be pathognomonic of a pulmonary exacerbation. However, it may be possible to develop a panel of markers that will increase diagnostic certainty and the ability to monitor the response of host and pathogen to treatment more reliably.

Epidemiology & impact of exacerbations

Data from patient registries demonstrate that the proportion of patients requiring intravenous antibiotics and hospital admissions for respiratory exacerbations increases with age, and is mirrored by a decline in lung function [40–42]. These findings are further supported by data from the Epidemiological Study of CF (ESCF), which reported that the rate of intravenous antibiotic use (at least one course per year) increased from only 23% in those aged under 6 years to 63% of patients aged over 18 years [14].

Pulmonary exacerbations have a significant impact on long-term lung health, with 25% of patients who suffer a pulmonary exacerbation failing to recover their baseline lung function following intravenous antibiotic treatment [17]. Furthermore children having a single, and adults having greater than three exacerbations in a year experience an accelerated decline in lung function in the subsequent three years [6]. These data are supported by a model predicting 5-year survival in CF, which demonstrated that each pulmonary exacerbation experienced annually was equivalent to a reduction in FEV_1 predicted of 12% [5]. Other impacts of exacerbations include an increased need for domiciliary oxygen, non-invasive ventilation and diminished health-related quality of life [7,43]. Severe pulmonary exacerbations necessitating intensive care unit (ICU) admission are associated with increased mortality, with in-hospital survival rates as low as 27% in patients requiring invasive ventilator support [43,44].

Given the clinical impact of pulmonary exacerbations in CF identification of factors that may predispose to their occurrence are important in planning preventative strategies. However, to date only one prospective study has examined factors that predict pulmonary exacerbations [45]. This study, performed in a cohort of patients chronically infected with multidrug resistant organisms (e.g., *P. aeruginosa*, *Burkholderia* spp) identified female sex, poor lung function, previous pulmonary exacerbations, and the use of inhaled corticosteroids as potential risk factors. Interestingly, a negative correlation between age and exacerbations was demonstrated, which the authors postulated may represent a survivor effect. A recent *post-hoc* analysis of data from the ESCF including 16,000 patients similarly identified decreased lung function, female sex, and previous exacerbations, as well as *P. aeruginosa* infection as significant risk factors for exacerbations regardless of age [46]. Other risk factors identified from smaller retrospective studies include, low socioeconomic status and acute viral infection [47–49].

Etiology of exacerbations

Pulmonary inflammation and infection begins a short time after birth [50,51]. Initial intermittent infection is often followed by the establishment of chronic infection, often with Gram-negative bacteria. Factors predisposing to the establishment of chronic infection are beyond the scope of this article, but excellent reviews of this topic are available [21,52].

Once chronic infection is established a vicious cycle of infection and inflammation develops and acute pulmonary exacerbations are presumed to occur when this balance is perturbed. Proposed mechanisms for the development of an exacerbation include acute respiratory viral infections, acquisition of a new bacterial strain, a change in the resident bacterial flora, or an acute increase in airway inflammation – potentially as a consequence of altered host immunity.

Virus induced exacerbations

An association between viral infections and pulmonary deterioration in CF was first described over 25 years ago [53]. In this sentinel study of children with CF, isolation of a virus at the time of a pulmonary exacerbation resulted in accelerated progression of lung disease. In subsequent studies, rates of viral isolation at the times of pulmonary exacerbations of between 9% and 46% have been described [48,54,55], with the highest reported rates in those studies that have used molecular detection techniques.

The viruses detected depend on the sampling technique (nasopharyngeal aspirate compared with cough swab and bronchoalveolar lavage) and the age of the study population. However,

the viruses are in general similar to those seen in people without CF, with rhinovirus, respiratory syncytial virus (RSV), influenza A and B, and para-influenza virus IV being the most commonly detected [48,54,55].

Debate exists about whether viruses trigger exacerbations in their own right, or act indirectly by increasing the virulence of existing airway bacteria. Possible mechanistic links between viral infection and bacterial virulence include virus-induced reduction in alveolar macrophage antibacterial responses and facilitation of bacterial binding to epithelial cells [56,57]. However other authors highlight the poor correlation between viral infection and increased bacterial number to argue that viruses have direct effects on airway inflammation [48]. There is also evidence that the anatomical site of viral infection within the airway may be important, for example lower respiratory tract viruses (including RSV and influenza) may influence the rate of lung function decline, whereas upper respiratory tract viruses (including rhinoviruses) appear to have less impact [55].

In 2009 the emergence of the first influenza pandemic to occur in the modern era of CF care gave an insight into how a novel viral infection may affect people with CF. Initial single center reports in the wake of the pandemic from Australia and UK reported that in general patients had mild clinical course [58,59]. Subsequently a large multinational study (24 European centers, one USA center) reported the outcomes in 110 cases from an at-risk population of greater than 4500 (incidence 2.3%). While most patients again suffered a self-limiting illness with a return to baseline lung function within 30 days of infection, there was significant short-term morbidity with two thirds requiring intravenous antibiotics and 50% being admitted to hospital. Patients with severe lung function impairment prior to infection were most severely affected, with six patients in this group requiring ICU admission and three dying [60].

Further work is required to delineate the pathogenic potential of specific viruses, including to determine the optimum sampling site and processing technique for diagnosis, and to investigate the role of antiviral agents in treatment of pulmonary exacerbations.

The role of chronic *P. aeruginosa* infection in exacerbations

By the age of 18 years, 80% of patients with CF will have chronic infection with *P. aeruginosa* [40,41]. Following its establishment, *P. aeruginosa* undergoes phenotypic and genetic adaptations that support persistent infection [61]. Arguably the most important of these is the transformation from a planktonic free-living state, to a 'mucoid' phenotype typified by the production of alginate, and the formation of bacterial communities encased within a 'biofilm' of extracellular glycoproteins. Such changes offer protection from host immune defenses and limit antibiotic penetration [62]. Biofilm dwelling bacteria are not eradicated by the host immune response, but their presence probably provides stimulus for chronic inflammation.

Biofilm dwelling bacteria employ density dependent cell-to-cell communication (QS) to ensure survival of the colony within the constraints of its environment. It has been proposed that relatively inert biofilms periodically expel colonies of immunogenic planktonic bacteria in response to environmental stimuli, which can induce an acute exacerbation [63]. This theory is supported by data showing that in 94% of patients with chronic *P. aeruginosa* infection the same strain is isolated during periods of clinical stability and acute exacerbations [64].

Culture negative exacerbations

In 20% of pulmonary exacerbations the typical bacteria of the CF airway are not detected, particularly in children [65]. Although some exacerbations may be caused by viruses, the etiology of the remainder are unexplained. Recent interest has focused on anaerobic bacterial pathogens as the polymicrobial nature of infection in the CF lung becomes apparent [32]. Thick mucus within the airways creates anaerobic and microaerobic niches and studies utilizing anaerobic culture and nonculture molecular techniques have provided compelling evidence that CF airways are chronically infected with anaerobic bacteria [32,33,66]. A number of the anaerobes that have been detected are known pulmonary pathogens responsible for nosocomial pneumonia and lung abscesses [67,68]. It is therefore possible that anaerobes, which are not detected by standard sputum culture techniques, play an important role in triggering pulmonary exacerbations. More studies are required to assess the clinical impact of anaerobes in the CF airway [33,69].

Fungi may also have a role in exacerbations. *Aspergillus* species are isolated in half of all CF sputum samples [70], and allergic bronchopulmonary aspergillosis (ABPA) is relatively common [71]. Although a 'flare' of ABPA may mimic a pulmonary exacerbation, it can usually be differentiated by the presence of airway

'casts', accompanied by elevated blood IgE, peripheral blood eosinophilia, and positive *Aspergillus* precipitins [71]. However, recent evidence demonstrating an increased rate of lung function decline and pulmonary exacerbations in patients with chronic *Aspergillus fumigatus* infection, independent of the formal diagnosis of ABPA suggests that airway fungal infection may have a pathogenic role in exacerbations [72].

Gastro-esophageal reflux disease (GORD) is common in CF, occurring in up to half of patients regardless of symptoms or acid suppression treatment [73]. Post lung transplantation, untreated GORD is associated with an increased rate of lung function decline and onset of chronic rejection [74], but its impact in CF prior to transplantation is poorly understood. There is evidence that GORD is associated with increased cough, and lung function decline [73], but the role of GORD in provoking exacerbations has not been studied. In CF patients who are 'frequent exacerbators', GORD may be an important contributor and should be actively sought and treated when present. Use of acid suppression treatment may neutralize the pH of fluid aspirated, but may also inadvertently increase the microbial burden of gastric fluid aspirated [75]. Further research is needed in this area, but early surgical fundoplication may be the preferred option when it can be performed safely [76].

Future perspective

Much is still unknown about the etiology of pulmonary exacerbations in CF. Future research will focus on identifying mechanisms by which infectious and noninfectious stimuli trigger an increase in the host immune response in order to identify common pathways that may be manipulated to reduce airway inflammation and remodeling.

The role of sputum microbiology in guiding the treatment of exacerbations

Traditional sputum microbiological techniques for routine CF respiratory samples use selective media to specifically culture known pathogenic bacteria, followed by determination of antibiotic susceptibility based on the minimum inhibitory concentration *in vitro*. When a single organism is responsible for pulmonary infection (e.g., community acquired pneumonia) susceptibility profiles are valuable in guiding antibiotic therapy [77,78]. However, CF pulmonary infection is polymicrobial and using routine culture and sensitivity results as a guide to antibiotic therapy may not be ideal. Culture-independent (metagenomic) microbiological techniques that utilize bacterial RNA present in specimens to generate bacterial profiles, suggest that culture-dependent techniques detect only a minority of the organisms that exist within the infective milieu of CF sputum [79]. Standard culture techniques also under-represent the phenotypic diversity among detected bacteria. Fothergill *et al.* have recently demonstrated immense heterogeneity in genotype and phenotype (including antibiotic resistance) between apparently identical morphotypes of *P. aeruginosa* cultured from a single sputum sample [34]. In comparison, current standard microbiological practices would only select a limited number of morphotypes for susceptibility testing, potentially resulting in a resistance profile that misrepresents the *P. aeruginosa* population as a 'whole' in an individual patient.

The efficacy of routine microbiology is also compromised by the difference in behavior of the most common CF pathogen, *P. aeruginosa* when growing in a biofilm compared with routine pathology laboratory culture systems. For instance biofilm dwelling *P. aeruginosa* are believed to be up to a 1000-times more resistant to antibiotics than planktonic growing bacteria [80].

These limitations of established antibiotic testing methods most likely explain the poor correlation between conventional susceptibility profiles generated by single agent antibiotic testing, and clinical response to treatment [81]. Interestingly, despite a lack of evidence of improved outcomes most clinical guidelines for CF care still advocate the use of antibiotic sensitivity profiles to select 'appropriate' antibiotic regimens [82,83].

Given the shortfalls of traditional single agent sensitivity testing methods a number of alternate strategies are currently being investigated. The recognition that a number of antibiotics exhibit synergistic effects when co-administered *in vitro*, has led to the development of techniques to test the sensitivity of *P. aeruginosa* to combinations of antibiotics [84]. However, application of these methods to guide clinical practice has been limited, with similar clinical and bacteriological responses observed in patients receiving treatment based on single agent sensitivity testing results compared to multiple combination bactericidal testing in a randomized control trial [85].

In vitro biofilm models for assessment of antibiotic susceptibility have been investigated [86,87]. To date, only one study has reported outcomes in patients with antibiotics selected by such techniques and this study also failed to demonstrate superior clinical or microbiological outcomes using the biofilm model [88]. Reasons for this failure may include the confounding effect of different environmental conditions in the lung that were not replicated within the biofilm model (e.g., oxygen concentration and pH) and the need to also account for the fact that a polymicrobial infection exists in the CF lung.

Future perspective

As we begin to more fully understand the complexity of airway infection in CF it is clear that novel microbiological diagnostic methods are required, which more accurately represent resident flora. The emergence and implementation of metagenomic techniques to characterize the bacterial diversity of airway secretion will hopefully improve our understanding of how polymicrobial infections respond to antibiotic treatment. The application of bioinformatics may also provide greater insight into the expression of bacterial resistance genes on a 'whole population' basis that will more accurately predict the response to treatment.

Treating pulmonary exacerbations

Antibiotics are used routinely to treat pulmonary exacerbations in patients with CF, yet this is based on limited evidence including only two small randomized clinical trials [89,90]. Despite neither study providing clear evidence of improved clinical end points in the antibiotic treatment arms, undertaking further placebo-controlled trials would be considered unethical, given the strong anecdotal evidence for benefit.

In selecting an antibiotic regime for an individual patient important considerations include, the specific bacterial pathogens present within the airways, preferred location of treatment (hospital vs home), route of administration, how many antibiotics to use, and which ones to prescribe [91]. These decisions are often influenced by exacerbation severity, available intravenous access, previous antibiotic allergies, adherence and the support available to the individual patient if home-based therapy is to be considered.

Bacteriological considerations

The choice of antibiotics is based on the sensitivity profile of bacteria present on culture of airway secretions. Early infection is typically with the common respiratory pathogen *Staphylococcus aureus*, and nontypeable *Haemophilus influenzae*, which are often replaced by *P. aeruginosa* in late childhood. Other Gram-negative bacteria that may cause chronic infection in a minority of patients include *Burkholderia cepacia* complex (BCC), *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans* [92].

The realization that chronic *P. aeruginosa* infection results in increased lung function decline, exacerbation frequency, morbidity and mortality has led to the development of early detection programs that often involve invasive bronchoscopies and aggressive antibiotic regimes aimed at eradicating *P. aeruginosa* infection at the time of first acquisition [93–96]. Implementation of these regimes within pediatric centers has proven to be effective in delaying the onset of chronic *P. aeruginosa* infection by several years [96], though their impact on the development of bronchiectasis is not well established [97]. However, the long-term impact on survival, antibiotic resistance profiles, and the potential acquisition of other opportunistic bacterial pathogens is not well understood. Similar strategies have been proposed for first acquisition of other CF airway pathogens, but again the long-term efficacy of these regimens remains to be proven (TABLE 2).

In recent years an evolution of airway bacterial infection has been witnessed with the emergence of several new opportunistic bacteria including *S. maltophilia*, *A. xylosoxidans*, methicillin-resistant *S. aureus* (MRSA), and other Gram-negative bacteria (e.g., *Ralstonia* and *Pandoraea* species), while rates of *Burkholderia* species infection have remained constant [92]. The explanation for the changing microbial profile is unclear but may include better microbiological detection techniques, the effects of more aggressive and successful treatment of *P. aeruginosa*, increased prevalence of the opportunistic pathogens within healthcare facilities, or simply a consequence of increased survival leading to selective pressure due to greater lifetime exposure to antibiotic therapy.

Whilst BCC infections are associated with worse clinical outcomes (including reduced post-transplant survival) [98–100], the clinical importance of other *Burkholderia* species and emerging Gram-negative pathogens are unknown. However, many of these other bacteria are inherently antibiotic resistant, which may increase their potential to significantly impact on disease course and survival in CF [101–103].

Table 2. Eradications strategies for treatment of specific airway pathogens at first isolation.

Bacteria	Antibiotic	Dose	Special instructions
Methicillin-sensitive *Staphylococcus aureus*	Flucloxacillin	100 mg/kg/day oral	Two agents in combination for 2–4 weeks. If persistent infection consider 2 weeks of combination iv. agents
	Fusidic acid	750 mg daily oral (> 12 years old) Dosing under 12 complicated see PI	
Methicillin-resistant *Staphylococcus aureus*	Topical mupirocin	Intranasal	Combined use of topical mupirocin (5 days) and two other oral agents for 6 weeks to 6 months
	Trimethorprim–sulfamethoxazole	160/800 mg b.i.d. oral	
	Fusidic acid	750 mg daily oral Dosing under 12 complicated see PI	
	Rifampicin	10 mg/kg/day oral Max 450 mg under 45 kg Max 600 mg over 45 kg	
Haemophilus influenzae	Amoxicillin–clavulanic acid	875/125 mg b.i.d. oral or 250/125 mg, 2 t.i.d. oral Dosing under 12 complicated see PI	Single agent for 2–4 weeks, repeated if still positive at the end of the course. Consider 2 week iv. course if remains positive
	Doxycycline	200 mg initial dose, 100 mg b.i.d. oral[†]	
	Cefaclor	500 mg t.i.d. (> 7 years), 250 mg t.i.d. (1–7 years) oral	
Pseudomonas aeruginosa	Ciprofloxacin	15 mg/kg oral b.i.d. (under 5 years) 20 mg/kg oral b.i.d. (5–18 years) 750 mg b.i.d. oral (adult)	3 weeks to 3 months of ciprofloxacin combined with an inhaled antibiotic Multiple regimens have been studied [91–96]
	Colistin	1 million units b.i.d. inhaled (under 2 years) 2 million units b.i.d. inhaled (over 2 years)	
	Tobramycin	300 mg b.i.d. inhaled	

[†]Avoid tetracyclines in patients under 12 years of age.
b.i.d.: twice daily; iv.: intravenous; Max: Maximum dose; PI: Product information; t.i.d.: Three-times daily.
Data taken from the report of the UK Cystic Fibrosis Trust Antibiotic Working Group, and the Australian Therapeutic Guidelines [82,83].

In the absence of Gram-negative organisms, choice of antibiotics is typically based on available microbiological cultures and sensitivity patterns, and single-agent treatment may be employed. In the presence of *P. aeruginosa* antibiotic therapy is directed towards this, and modified to target co-infections if clinical response is suboptimal (Table 3).

Antipseudomonal antibiotic classes

Antibiotic options for treatment of *P. aeruginosa* are limited (Table 4). *Fluoroquinolones* exert their bactericidal effect against Gram-negative bacteria through inhibition of DNA gyrase and remain the only oral agent to which *P. aeruginosa* is susceptible *in vitro*.

Aminoglycosides demonstrate dose-dependent antimicrobial effects, suggesting the peak plasma concentration achieved is the most important pharmacokinetic attribute for bacterial killing. This is supported by studies demonstrating the equivalence of once-daily administration compared with traditional multidose regimes [104,105]. The major limitation of systemically administered aminoglycosides is the potential to develop serious and permanent toxic side effects in patients with CF including nephrotoxicity, vestibular disturbance, and ototoxicity (rates of 42, 30 and 17%, respectively) resulting from long-term cumulative exposure [106–108]. Choice of individual aminoglycoside and co-administration of another antipseudomonal agent are important in limiting their toxic effects, with tobramycin being associated with less nephrotoxicity than gentamicin [109]. Similarly, the combined nephrotoxic effects of tobramycin and colistimethate sodium are greater than either agent administered alone [106]. Other factors that may limit nephrotoxicity include, once-daily dosing regimens, and morning aminoglycoside dosing [104]. Therefore despite antibiotic guidelines typically suggesting a total tobramycin dose of 10 mg/kg, careful monitoring of plasma levels is imperative to prevent both acute and long-term toxicity. The dose prescribed may often need to be substantially reduced, particularly in older patients and doses may need to be varied between admission, as well as over time. Several models for aminoglycoside monitoring have been developed, however, there is currently no consensus on the best method to employ [110].

β-lactam antibiotics (β-lactams), in contrast to aminoglycosides exert their bactericidal effects through time-dependent inhibition of bacterial cell wall synthesis, suggesting maintaining plasma levels above the minimal inhibitory

concentration (MIC) of the bacterial pathogen may be important. This led to the hypothesis that continuous infusions would have greater antibacterial effects than intermittent dosing regimens. However, to date **β-lactams delivered** by continuous infusion have not proven more efficacious than multiple daily doses [111]. In addition a recent study suggested that twice daily ceftazadime dosing may be equivalent to traditional three-time daily dosing [112], although the study has been criticized for being underpowered [113].

There are several classes of **β-lactams including**, the extended spectrum penicillins (e.g., ticarcillin and piperacillin) and third generation cephalosporins (ceftazadime), which together are the most extensively studied and widely used first-line agents [114], but are also associated

Table 3. Oral and intravenous antibiotics for the treatment of pulmonary exacerbations where *Pseudomonas aeruginosa* is not isolated[a].

Bacteria	Antibiotic	Dose	Special considerations
Oral antibiotics			
Methicillin-sensitive *Staphylococcus aureus*	Di/Flucloxacillin	125 mg b.i.d. (prophylaxis) 25 mg/kg q.d. (<18 years of age) 1–2 g q.d.	Consider prophylaxis in 0–3 years of age, but currently unproven clinical benefit
	Trimethorprim–sulfamethoxazole	160/800 mg b.i.d.	
	Clindamycin	450 mg t.i.d.	
	Roxithromycin	150 mg b.i.d.	
Stenotrophomonas maltophilia	Trimethorprim–sulfamethoxazole	160/800 mg b.i.d.	Targeted treatment currently of unproven benefit
	Minocycline	200 mg initial dose, 100 mg b.i.d.[b]	
	Tigecycline	100 mg initial dose, 50 mg b.i.d.[b]	
Intravenous antibiotics			
Methicillin-sensitive *Staphylococcus aureus*	Di/flucloxacillin	50 mg/kg (<18 years) 500 mg to 1 g q.d.	
Methicillin-resistant *Staphylococcus aureus*	Vancomycin[c]	1 g b.i.d.	
	Teicoplanin	10 mg/kg b.i.d. three doses, then daily (<18 years) 400 mg for b.i.d. three doses, then daily	
	Linezolid	10 mg/kg (<12 years) 600 mg daily	
Haemophilus influenzae	Cefuroxime	50 mg/kg t.i.d. (1–18 years old) 1.5 g t.i.d.	
	Cefotaxime	50 mg/kg t.i.d. (1–18 years of age) 2 g t.i.d.	
Burkholderia cepacia complex	Ceftazidime	50 mg/kg t.i.d. (1–18 years of age) 2–3 g t.i.d.	A combination of at least two antibiotics from different classes should be used
	Meropenem	25–40 mg/kg t.i.d. (4–18 years of age) 2 g t.i.d.	
	Imipenem	22.5 mg/kg t.i.d. (<40 kg) 1 g t.i.d.	
	Piperacillin–tazobactam	90 mg/kg q.d. (children) 4.5 g q.d.	
	Trimethorprim–sulfamethoxazole	240 mg b.i.d. (6 months to 6 years of age) 480 mg b.i.d. (6–12 years of age) 960 mg b.i.d. (>12 years of age)	
	Tobramycin[c]	10 mg/kg daily	Plasma concentration monitoring required
Stenotrophomonas maltophilia	Ticarcillin–clavulanate	80–100 mg/kg q.d. (<18 years) 3.1 g q.d.	

This table is intended as a guide only, antibiotic choice may vary dependant on local resistance profiles and antibiotic availability.
[c]Therapeutic drug monitoring is required.
[b]Avoid tetracyclines in patients under 12 years of age.
b.i.d.: Twice daily; Max: Maximum dose; q.d.: Four-times daily; t.i.d.: Three-times daily.
Data taken from the Report of the UK Cystic Fibrosis Trust Antibiotic Working Group, and the Australian Therapeutic Guidelines [82,83].

with high rates of allergy [115]. Carbapenams (meropenem and imipenem) and fourth generation cephalosporins (cefepime) display activity against both Gram-positive and Gram-negative bacteria, suggesting they may have utility in treatment of co-infection with *S. aureus* and *P. aeruginosa* [116]. Meropenem has demonstrated equivalence to ceftazadime containing regimes in treating pulmonary exacerbations, with lower rates of *P. aeruginosa* resistance developing [117,118]. However, the recent emergence in CF of *P. aeruginosa* mutants producing metal- **β-lactamases capable of inactivating** all clinical β-lactam substrates (with the exception of aztreonam) raises concerns that increasing resistance rates will be seen in the future [119]. Meropenem also has high *in vitro* activity against anaerobic bacteria present in the CF airway secretions, suggesting it may have additional benefits compared to other antipseudomonal agents in the setting of polymicrobial infection [32]. There are no comparative trials of the cefepime to other treatment regimes in CF, and higher rates of *in vitro P. aeruginosa* resistance have been reported in comparison to ceftazidime [120]. The monobactam antibiotic, aztreonam, has a spectrum of activity limited to Gram-negative organisms. Aztreonam

Table 4. Currently licensed antibiotics for the treatment of *Pseudomonas aeruginosa* in cystic fibrosis.

Class	Drug	Route of administration	Dose	Special considerations
β-lactams				
Extended spectrum penicillins	Ticarcillin/clavulanate[†]	Intravenous	80–100 mg/kg q.d. (child) 3.1 g q.d. (adult)	Need to be administered by slow intravenous infusion
	Piperacillin/tazobactam[†]	Intravenous	90 mg/kg q.d. (child) 4.5 g q.d. (adult)	
Third generation cephalosporin	Ceftazidime	Intravenous	50 mg/kg t.i.d. (under 18 years) 3 g t.i.d. (adult)	
Carbapenems	Meropenem[†]	Intravenous	25–40 mg/kg t.i.d. (4–18 years old) 2 g t.i.d. (adult)	
	Imipenem[†]	Intravenous	22.5 mg/kg q.d. (under 40 kg) 1 g t.i.d. (over 40 kg)	Need to be administered by slow intravenous infusion
Monobactam	Aztreonam	Intravenous	30 mg/kg (under 2 years) 50 mg/kg (2–12 years) 2 g t.i.d. (over 12 years)	
		Inhaled[‡]	75 mg t.i.d.	US FDA approved
Fourth generation cephalosporin	Cefepime	Intravenous	50 mg/kg b.i.d. (under 40 kg) 2 g b.i.d. (over 40 kg)	
Aminoglycosides				
	Tobramycin	Intravenous	10 mg/kg daily[§]	Plasma concentration monitoring required
	Tobramycin inhalation solution	Inhaled[‡]	300 mg b.i.d.	
Fluoroquinolones				
	Ciprofloxacin	Oral	15 mg/kg (under 5 years) 20 mg/kg (5–18 years) 750 mg b.i.d. (adult)	Mild exacerbations and eradication regimens
Polymyxin B				
	Colistin	Intravenous	25000 units/kg t.i.d. (under 60 kg) 2 million units t.i.d. (over 60 kg)	Need to be administered by slow intravenous infusion
		Inhaled[‡]	1 million units b.i.d. (under 2 years) 2 million units b.i.d. (over 2 years)	
Other agents				
	Fosfomycin	Intravenous	100 mg/kg (under 40 kg) 5 g t.i.d. (over 40 kg)	

[†]Co-administration of probenecid may be considered to increase drug levels.
[‡]Have not been studied in treatment of exacerbations.
[§]Therapeutic drug monitoring is required.
b.i.d.: Twice daily; q.d.: Four-times daily; t.i.d.: Three-times daily.

contains an unfused β-lactam ring that confers low cross-reactivity with other β-lactam drugs meaning that it can be used safely in patient with penicillin hypersensitivity [121].

Colistimethate sodium (colistin) was originally discovered in the 1940s, but seldom used for several decades due to concerns about its adverse effects. However, it has re-emerged as an antibiotic in patients with CF due to its activity against Gram-negative bacteria including multidrug resistant *P. aeruginosa*. The major adverse effects limiting its use are nephro- and neuro-toxicity [122]. Parenterally administered colistin may be most suited for patients with multidrug resistant *P. aeruginosa*, or in patients where aminoglycosides are contraindicated due to previous toxicity.

Fosfomycin is another 'forgotten' drug with efficacy against multidrug resistant *P. aeruginosa*. Despite evidence for its clinical efficacy in CF being limited to cohort and case studies only [123], *in vitro* data demonstrating its ability to penetrate biofilms, and to act synergistically with agents from each of the major classes of antipseudomonal antibiotics [124], combined with a benign side effect profile suggest it is a useful second-line agent [125]. However, potential for resistance to develop suggests its use is best used only in patients with limited antibiotic options due to allergies and toxicity of other antibiotic agents [126].

Where, how & which antibiotic?

During a severe pulmonary exacerbation, a patient's clinical status should dictate the need for hospital admission. However, many patients with milder exacerbations prefer home-based treatment, which reduces the impact on work and family commitments [127]. When comparing home with hospital treatment the available small studies present conflicting evidence. The equivalent outcomes and improved quality of life from home-based treatment demonstrated in some studies [127,128] needs to be considered in the context of other studies that have demonstrated greater improvements in lung function and weight with hospital-based treatment, as well as a reduction in the duration of therapy [129,130]. A recent, large (>1500 patients) retrospective study has supported home-based treatment demonstrating that although hospital-treated patients appear to gain greater improvement in lung function in the immediate post-treatment period, long-term outcomes, and time to next exacerbation were similar regardless of treatment location [131].

Once treatment location is decided the next consideration is how to administer therapy. As discussed previously, fluoroquinolones are the only orally administered bioavailable antipseudomonal agents. Ciprofloxacin is the most commonly prescribed quinolone, and is frequently combined with a nebulized agent in outpatient treatment regimens for mild exacerbations. Nebulization allows delivery of high drug concentration directly to the airway whilst avoiding systemic adverse effects. Unfortunately inhaled administration of antibiotics fails to treat recalcitrant bacteria sequestered in under-ventilated lung regions. Until recently aminoglycosides and colistin were the only nebulized agents in clinical use. However, multicenter Phase III randomized controlled trials (RCTs) have recently demonstrated the safety and efficacy of aerosolized aztreonam lysine in stable patients with moderate-to-severe lung function impairment [132,133], which has supported US FDA approval for use as a maintenance treatment in CF.

Despite oral and nebulized combination regimes often being used in clinical practice, there is limited evidence to support their use in the acute setting. A Cochrane review identified three small randomized controlled trials comparing ciprofloxacin monotherapy with combination intravenous therapy. No major differences in outcome were seen, but each of the trials was under-powered and the treatment arms were not blinded [134].

Intravenous antibiotic regimes remain the cornerstone of treatment of severe exacerbations in hospitalized patients. Intravenous therapy may also be delivered effectively in the home, providing patients have reliable intravenous access, are appropriately educated in self-administration and have access to 24 h medical support should difficulties arise.

Further considerations when administering intravenous antibiotics-in-the-home setting include the mode of delivery (infusion vs slow intravenous injection) and the stability of the agents in solution. A number of antibiotics demonstrate limited stability in solution (e.g., meropenem) and the patient must be educated in the sterile preparation of these prior to each administration. To overcome the obstacle of slow intravenous infusion of medication a number of small, portable pressure driven devices have been designed, which deliver a fixed rate of drug delivery and negate the inconvenience of infusion pumps and gravity driven delivery.

The superiority of combination intravenous therapy for Gram-negative nosocomial

pneumonia has been clearly demonstrated [135]. However, in the setting of chronic pulmonary infection in CF, systematic reviews comparing combination with monotherapy have failed to clearly demonstrate superiority of combination therapy [114,136,137]. However, whilst acknowledging the current lack of evidence, treatment guidelines recommend dual antipseudomonal antibiotic therapy [82,137]. No combination regimen has demonstrated clear superiority over the others, but in practice two agents from different antimicrobial classes (usually a β-lactam and aminoglycoside) are typically administered.

In selecting specific agents current practice guidelines recommend reference be made to available sensitivity profiles, and also recommend avoiding agents to which organisms are resistant [82]. However given the previously discussed limitations of routine sensitivity testing, and their poor correlation with clinical response, an alternate approach is to select antibiotics to which the individual patient has previously responded. Anecdotally, clinicians may only refer to antibiotic sensitivity profiles when second-line agents are being selected, in the situation where the patient has failed to improve with initial treatment regimen.

Finally, lifetime cumulative exposure to antibiotics results in rates of β-lactam allergy of up to three-times that of the general population, which limits treatment options in a significant proportion of patients. In work from our group, Burrows *et al.* demonstrated a prevalence of 36% for allergy to a single class of β-lactam antibiotic, and 19% of patients had multiple β-lactam allergies [115]. In some patients with multiple antibiotic allergies hospital-based desensitization protocols allow the safe re-introduction of β-lactams to which they have previously reacted [138]. However, recurrent allergy is common and patients must be closely monitored. The additional burden is that desensitization must be repeated for each individual treatment course.

Duration of therapy

There are no RCTs studying the optimum duration of antibiotic therapy for pulmonary exacerbations in CF [137,139]. Theoretical concerns that a shorter duration of antibiotic therapy may reduce the time to the next exacerbation, or increase the rate of lung function decline, need to be balanced against the potential adverse effects [2], and risk of promoting antibiotic-resistant organisms [4], which may be associated with prolonged treatment courses.

Prospective studies in the treatment of acute exacerbations of chronic bronchitis, and microbiologically proven ventilator-associated pneumonia (VAP) have demonstrated noninferiority of shorter courses of antibiotics [140,141]. Although, of note in the case of VAP, patients infected with Gram-negative organisms (commonly *P. aeruginosa*) had an increased risk of microbiological relapse with shorter treatment [141].

In a small prospective cohort study of 22 CF patients undergoing hospital-based treatment for pulmonary exacerbations, improvements in spirometry and oxygenation plateaued after 8 days of treatment [142]. These limited prospective data are supported by a recent large retrospective study of outcomes following pulmonary exacerbations in over 1500 people, which suggested that further improvements in spirometry after 8–10 days of therapy were small, and that shortening antibiotic duration did not adversely affect the time to next exacerbation. Adequately powered prospective studies are required to more fully understand the optimal duration of treatment for pulmonary exacerbations in CF [131].

New antipseudomonal antibiotic options

Development of new antibiotic agents over the past two decades has been limited. Factors that contribute to the reluctance of pharmaceutical companies to invest in antibiotic development include microbiological (e.g., bacterial adaptability leading to resistance while drugs are still in development), regulatory (e.g., need to demonstrate superiority to other agents prior to licensing) and financial (greater profitability in development of other pharmaceuticals e.g., anticancer or immunomodulatory agents) [143,144]. Therefore, recent developments have instead focused on repackaging existing agents, for example for inhalation delivery. Alongside the recently licensed inhaled aztreonam, other inhalational preparation undergoing investigation in RCTs (both Phase II and III) include levofloxacin, coformulation of fosfomycin and tobramycin, liposomal amikacin, and dry powder formulations of tobramycin, colistin and ciprofloxacin [145,146]. Although these medications are being developed primarily for use in maintenance regimens they have the potential to be studied for use in the treatment of pulmonary exacerbations.

Novel targets for drug development

The formation of a biofilm that offers physical protection to *P. aeruginosa* from host immune clearance and antimicrobial agents represents a

significant challenge to developers of new antipseudomonal agents. In an attempt to meet this challenge the focus of research has moved away from bactericidal agents to the development of agents capable of disrupting biofilms, or agents that limit biofilm dwelling bacteria access to essential nutrients.

Quorum sensing is essential to the development of robust biofilms, therefore anti-QS agents are of major interest. A recent study has reported the ability of garlic to block the production of QS molecules by *P. aeruginosa in vitro*, and disrupt biofilm development in mouse urinary and respiratory tract infection models [147,148]. A pilot placebo-controlled study has demonstrated safety and tolerability of garlic in patients with CF, but did not show any differences in outcomes compared to controls [149].

Biofilm 'dispersal' is a naturally occurring process that allows propagation of infection to other sites when available nutrients in the local environment are sufficient to support an expansion in the bacterial community. During dispersal biofilms liberate planktonic bacteria in response to environmental triggers, leaving behind an empty extracellular matrix shell. Given that *in vitro* studies suggest planktonic bacteria are significantly more susceptible to antibiotics than their biofilm dwelling counterparts, it is theoretically possible that inducing dispersal in the presence of antibiotics will enhance their bactericidal effects. Overall control of dispersal is again under the control of QS, but both reactive oxygen and nitrogen species are capable of triggering dispersal [150]. *In vitro* experiments have demonstrated the ability of nontoxic levels of sodium nitroprusside (a nitric oxide donor) to trigger dispersal and increase antibacterial killing when combined with antipseudomonal antibiotics. To date there have been no human trials of dispersal agents.

Iron is an essential nutrient to *P. aeruginosa* and the organism has become adept at scavenging iron from the environment [151]. The primary mechanism by which it obtains iron is through the production of secreted molecules with high iron binding coefficients that are capable of sequestering iron from the environment (siderophores), and delivering it to the bacterium. Interestingly, culturing *P. aeruginosa* in either iron-depleted medium, or medium containing excess iron leads to failure of robust biofilms and disruption of established biofilms [152–154]. Similar results are seen when naturally occurring and synthetic iron chelators are utilized as iron competitors [153].

A novel approach has been to conjugate antibiotics to siderophores as a 'Trojan horse' approach to delivering antibiotics to biofilm dwelling bacteria. These studies are in their infancy, but have shown encouraging results [155].

The studies discussed above offer exciting insights into potentially clinical useful antibiofilms agents. However, for the most part this research has been performed using laboratory strains of *P. aeruginosa*, which may differ considerably in their behavior compared with clinically relevant strains, and in *in vitro* biofilms models that poorly reflect the environmental conditions present in the CF lung.

Future perspective

Further research examining short course antibiotic regimens for treatment of pulmonary exacerbations is merited. However, the lack of defined end points to signal the end of an exacerbation, and reluctance of physicians (and patients) to deviate from current 'successful' treatment regimens will make studies difficult to design and successfully complete.

Future development of novel antibacterial agents will require assessment of their efficacy against clinical *P. aeruginosa* strains, in models developed to more accurately represent conditions in the CF airways. Given that planktonic bacteria released from biofilms are theoretically more immunogenic than their biofilm counterparts, it will be important to carefully assess any potential adverse effects of these strategies on the host inflammatory response.

Adjunctive therapies to prevent & treat pulmonary exacerbations

The proven benefits of oral corticosteroids in exacerbations of asthma and COPD have led to their use in CF exacerbations being proposed. Only one RCT has been performed to examine the effects of oral corticosteroids in CF [156]. This pilot placebo-controlled study examining the effect of a 5-day course of oral prednisolone in 24 children with CF undergoing hospital treatment for a pulmonary exacerbation demonstrated a modest (3.6%), nonsignificant improvement in FEV_1 at 14 days, but this was at the expense of increased rates of treatment related adverse events. The authors concluded that more than 250 patients are needed to adequately power a trial to detect a 4% improvement in FEV_1. Despite this lack of published evidence, a survey of UK CF physicians has suggested that many will use corticosteroids on a case-by-case basis [157].

Given the significant deleterious effects and increased healthcare costs associated with pulmonary exacerbations effective utilization of maintenance treatments to prevent their occurrence is paramount [158]. Much of the improvement seen in the health of people with CF has coincided with the development of multidisciplinary CF care teams, and wherever feasible patient care should be coordinated by a CF specialist center [159,160].

Maintenance exercise and physiotherapy regimens are vital in assisting clearance of viscous airway secretions. A number of chest physiotherapy techniques, including active cycle of breathing, percussion and autogenic drainage, as well as a range of hand-held expiratory oscillatory devices are available to enhance airway clearance, but superiority of one method over the others has not been established, and the individual method employed is usually negotiated between the patient and the expert physiotherapist [161]. Despite widespread acknowledgment of the importance of physiotherapy in maintaining lung health in CF, there are few studies that report on the impact on prevention of, and recovery from pulmonary exacerbations [158].

Inhaled mucolytic and rehydrating agents including recombinant DNAse (Dornase alfa), hypertonic saline and mannitol further facilitate sputum clearance, and have an established role in maintenance regimens [162–164]. Although the role of these agents in the treatment of exacerbations has not been established, pulmonary exacerbations are typically accompanied by an increase in volume and purulence of respiratory secretions, and clearance of these secretions is important in resolution of exacerbations. Therefore maintenance mucolytic regimens should be continued and where tolerated, intensified during exacerbations [137]. However, in clinical practice, tolerance of hypertonic saline and mannitol may be reduced during periods of heightened airway inflammation, especially in patients with severe lung disease.

The use continuous or cyclical courses of antibiotics have been extensively investigated as a method of preventing lung function decline. CF specialist centers in Denmark attribute increased survival in their patients to administration of regular parenteral antibiotics, irrespective of clinical status [165]. However, clinical trials have yet to show superiority of this approach over prescribing antibiotics based on symptoms [114,166]. An alternate approach is to use regular inhaled antibiotics either continuously or on alternate months. This approach was subject to a recent Cochrane review that identified 17 RCTs comparing maintenance inhaled antibiotic regimens to placebo. The review concluded that inhaled antibiotic use was associated with improved lung function, and a reduction in exacerbation rates, but increased rates of resistant *P. aeruginosa* were observed in the antibiotic treated patients [167]. Oral maintenance anti-staphylococcal treatment has been widely prescribed in young children in an attempt to prevent *S. aureus* infection and lung damage. Such regimens reduce rates of *S. aureus* isolation but beneficial effects on lung function or exacerbation rates have not yet been seen, and concern exists that these regimens may result in increased rates of *P. aeruginosa* acquisition [168]. Use of oral azithromycin has been shown to improve lung function and reduce exacerbations rates, particularly in patients with chronic *P. aeruginosa* infection and should be continued during pulmonary exacerbations [15,169,170].

Noninvasive ventilation (NIV) is an emerging therapy in CF [171], which may be used as a physiotherapy adjunct, or as a means of short-term ventilatory support. The need for invasive mechanical ventilation (IMV) during a pulmonary exacerbation is associated with poor survival, and IMV is a contraindication to acute lung transplantation in many centers [43]. NIV has been proposed as an adjunct to prevent the need for IMV. Although there are no RCTs comparing NIV to IMV in CF, observational studies suggest NIV treatment is associated with better outcomes during pulmonary exacerbations and can be used as a bridge to lung transplantation in patients with chronic respiratory failure [44,172–174]. These studies are likely subject to selection bias, but do support a trial of NIV in exacerbations complicated by respiratory failure. As an adjunct to physiotherapy, NIV has been shown to reduce hypoxia and improve tolerance of treatment [175].

Malnutrition is common in CF, and results in reduced lung function and survival [5,176]. Improving nutritional status using high-energy/high-protein supplements may prevent deterioration in lung function [177,178]. However, addressing dietary issues is often difficult and requires expert dietetic, nursing and psychological input in order to not only institute nutritional supplements, but also engender the behavioral changes required to maintain modifications over a prolonged period. The challenge of maintaining weight is added to by a further worsening of the already increased resting energy expenditure present in CF [142], which is often accompanied by a decrease in appetite and anorexia, as well as nausea related to antibiotics. In this setting, although oral supplements

are typically used in the first instance, enteral feeding may also need to be considered [179].

Conclusion

Despite the recognized importance of pulmonary exacerbations in lung function decline and reduced survival in CF, much is still unknown about their etiology, and treatment is still largely based on expert opinion and experience.

Future perspective

It is hoped that the emergence of molecular techniques capable of more accurately representing the bacterial diversity within respiratory secretions, a greater understanding of the biofilm model of bacterial communities, and the development of techniques to test antibacterial agents in *in vitro* models that mimic more closely conditions in the CF airways will herald advances in the treatment of exacerbations.

The polymicrobial nature of infection adds to the complexity in decision making in the treatment of pulmonary exacerbations and will make studies of antibiotic effectiveness more complex, and harder to interpret. In the absence of new classes of antibiotics, novel approaches to airway infection in CF will be required to ensure ongoing improvements in the health of people with CF.

Executive summary

- Acute pulmonary exacerbations contribute to diminished quality of life, reduced lung function, morbidity and mortality in cystic fibrosis (CF), and remain a major obstacle to further improving survival.

Definition

- The lack of a universally accepted definition of a pulmonary exacerbation prevents direct comparisons being made between clinical trials.
- Future directions:
  - Identification of biomarkers reflective of clinical status may facilitate the creation of a standard definition for application in research and clinical practice.

Epidemiology

- Risk factors for pulmonary exacerbations include previous exacerbations, reduced lung function, female sex, and chronic *Pseudomonas aeruginosa* infection.
- Future directions:
  - Monitoring the changes in the epidemiology of airway infection, including emerging bacterial pathogens patterns will be crucial in monitoring response to new therapeutic strategies.

Etiology

- Acute exacerbations occur when the balance between chronic bacterial infection and host immunity is perturbed. Some exacerbations are triggered by acute bacterial or viral infections, but in many cases the cause is unknown.
- Future directions:
  - Identification of common pathways by which infectious and noninfectious stimuli trigger acute exacerbations may allow development of novel therapeutic agents.

Microbiology

- Current culture-dependent microbiological techniques are inadequate to identify the diversity of bacteria present in the CF airway, and poorly predict response to treatment in polymicrobial and biofilm infections that frequently occur.
- Future directions:
  - Culture independent microbiological (metagenomic) techniques will more accurately represent bacteria present in CF airway infection.
  - Application of bioinformatic approaches to identification of bacterial resistance genes may allow more accurate prediction of treatment response.

Treatment

- Many aspects of treatment, including the optimum number and combination of antibiotics to use, and duration and location of treatment, are based on very limited evidence. Antibiotic options for treatment of chronic Gram-negative bacterial infections are limited, and further impacted on by development of bacterial resistance and antibiotic allergies.
- Future directions:
  - Prospective studies comparing outcomes from 'short-course' antibiotic regimens may allow reduction in cumulative antibiotic exposure.
  - Novel antibacterial agents will target biofilm growth and bacterial access to essential nutrients.

Adjunctive treatment

- Treatment in specialist centers by a multidisciplinary team including specialist nurses, physiotherapists and dieticians has significantly contributed to improved survival in CF. Adherence to maintenance therapies including physiotherapy, mucolytics and nutritional supplements are important in preventing exacerbations. Maintenance and eradication antibiotic regimens delay acquisition of chronic bacterial pathogens, but their long-term impact on outcome is as yet unknown.
- Future directions:
  - Surveillance of patients treated with *Pseudomonas aeruginosa* eradications regimens, *Staphylococcus aureus* prophylaxis, and chronic azithromycin therapy will determine the impact of these interventions on bacterial infections and long-term outcomes.

Financial & competing interests disclosure

SC Bell has received grant supports from the NHMRC and from Queensland Health. Bell has also received support to undertake clinical trials by the following: Vertex, Boehringer Ingelheim, Aradigm and Mpex, in the past 5 years. He has served on advisory boards to provide advice for clinical trial design and conduct with the following: Boehringer Ingelheim and Mpex, and is also a member of the Publications Committee for an ongoing clinical trial for Vertex. DW Reid has received grant supports from the NHMRC and Queensland Health. He has received a one-off grant from Novartis Pharmaceuticals within the past 5 years to undertake an investigator initiated study. DJ Smith has received a research scholarship from the School of Medicine (Northside Clinical School – The Prince Charles Hospital), University of Queensland. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Bibliography

Papers of special note have been highlighted as:
- \- of interest
- \-- of considerable interest

▶1 O'Sullivan BP, Freedman SD. Cystic fibrosis. *Lancet* 373(9678), 1891–1904 (2009).

▶2 Parmar JS, Nasser S. Antibiotic allergy in cystic fibrosis. *Thorax* 60(6), 517–520 (2005).

▶3 Prayle A, Watson A, Fortnum H, Smyth A. Side effects of aminoglycosides on the kidney, ear and balance in cystic fibrosis. *Thorax* 65(7), 654–658 (2010).

▶4 Merlo CA, Boyle MP, Diener-West M, Marshall BC, Goss CH, Lechtzin N. Incidence and risk factors for multiple antibiotic-resistant *Pseudomonas aeruginosa* in cystic fibrosis. *Chest* 132(2), 562–568 (2007).

▶5 Liou TG, Adler FR, Fitzsimmons SC, Cahill BC, Hibbs JR, Marshall BC. Predictive 5-year survivorship model of cystic fibrosis. *Am. J. Epidemiol.* 153(4), 345–352 (2001).

▶6 Sanders DB, Bittner RC, Rosenfeld M, Redding GJ, Goss CH. Pulmonary exacerbations are associated with subsequent FEV_1 decline in both adults and children with cystic fibrosis. *Pediatr. Pulmonol.* 46(4), 393–400 (2011).

\- Retrospective study providing strong evidence in support of the association between acute pulmonary exacerbations and accelerated lung function decline.

▶7 Britto MT, Kotagal UR, Hornung RW, Atherton HD, Tsevat J, Wilmott RW. Impact of recent pulmonary exacerbations on quality of life in patients with cystic fibrosis. *Chest* 121(1), 64–72 (2002).

▶8 Lieu TA, Ray GT, Farmer G, Shay GF. The cost of medical care for patients with cystic fibrosis in a health maintenance organization. *Pediatrics* 103(6), e72 (1999).

▶9 Ramsey BW, Boat TF. Outcome measures for clinical trials in cystic fibrosis: summary of a cystic fibrosis foundation consensus conference. *J. Pediatr.* 124(2), 177–192 (1994).

\- Conference report addressing the need to adopt universally recognized outcome measures for use within clinical trials in cystic fibrosis, including a singularly accepted definition of what constitutes a pulmonary exacerbation.

▶10 Sagel SD, Chmiel JF, Konstan MW. Sputum biomarkers of inflammation in cystic fibrosis lung disease. *Proc. Am. Thorac. Soc.* 4(4), 406–417 (2007).

\- Comprehensive review of the literature to date on the use of biomarkers detected in respiratory secretions to monitor disease severity and response to treatment, with a particular focus on current limitations and future research directions.

▶11 Johnson C, Butler SM, Konstan MW, Morgan W, Wohl ME. Factors influencing outcomes in cystic fibrosis: a center-based analysis. *Chest* 123(1), 20–27 (2003).

▶12 Dakin C, Henry RL, Field P, Morton J. Defining an exacerbation of pulmonary disease in cystic fibrosis. *Pediatr. Pulmonol.* 31(6), 436–442 (2001).

▶13 Rosenfeld M, Emerson J, Williams-Warren J *et al.* Defining a pulmonary exacerbation in cystic fibrosis. *J. Pediatr.* 139(3), 359–365 (2001).

\-- Prospective study in which the authors use responses to a physician completed questionnaire to develop a tool for predicting the presence of pulmonary exacerbations based on patient symptoms and lung function.

▶14 Rabin HR, Butler SM, Wohl ME *et al.* Pulmonary exacerbations in cystic fibrosis. *Pediatr. Pulmonol.* 37(5), 400–406 (2004).

▶15 Wolter J, Seeney S, Bell S, Bowler S, Masel P, Mccormack J. Effect of long term treatment with azithromycin on disease parameters in cystic fibrosis: a randomised trial. *Thorax* 57(3), 212–216 (2002).

▶16 Fuchs HJ, Borowitz DS, Christiansen DH *et al.* Effect of aerosolized recombinant human DNase on exacerbations of respiratory symptoms and on pulmonary function in patients with cystic fibrosis. The pulmozyme study group. *N. Engl. J. Med.* 331(10), 637–642 (1994).

▶17 Sanders DB, Hoffman LR, Emerson J *et al.* Return of FEV_1 after pulmonary exacerbation in children with cystic fibrosis. *Pediatr. Pulmonol.* 45(2), 127–134 (2010).

\-- Study reports upon the failure of lung function to return to baseline in 25% of patients following intravenous antibiotics, with degree of reduction in lung prior to exacerbation being most predictive of failure to recover, suggesting early intervention may improve long term outcomes.

▶18 Colombo C, Costantini D, Rocchi A *et al.* Cytokine levels in sputum of cystic fibrosis patients before and after antibiotic therapy. *Pediatr. Pulmonol.* 40(1), 15–21 (2005).

▶19 Gray RD, Imrie M, Boyd AC, Porteous D, Innes JA, Greening AP. Sputum and serum calprotectin are useful biomarkers during CF exacerbation. *J. Cyst. Fib.* 9(3), 193–198 (2010).

▶20 Laguna TA, Wagner BD, Luckey HK *et al.* Sputum desmosine during hospital admission for pulmonary exacerbation in cystic fibrosis. *Chest* 136(6), 1561–1568 (2009).

▶21 Elizur A, Cannon CL, Ferkol TW. Airway inflammation in cystic fibrosis. *Chest* 133(2), 489–495 (2008).

▶22 Chiron R, Grumbach YY, Quynh NV, Verriere V, Urbach V. Lipoxin A(4) and interleukin-8 levels in cystic fibrosis sputum after antibiotherapy. *J. Cyst. Fib.* 7(6), 463–468 (2008).

▶23 Brockbank S, Downey D, Elborn JS, Ennis M. Effect of cystic fibrosis exacerbations on neutrophil function. *Int. Immunopharmacol.* 5(3), 601–608 (2005).

▶24 Downey DG, Brockbank S, Martin SL, Ennis M, Elborn JS. The effect of treatment of cystic fibrosis pulmonary exacerbations on airways and systemic inflammation. *Pediatr. Pulmonol.* 42(8), 729–735 (2007).

▶25 Van Rheenen PF, Van De Vijver E, Fidler V. Faecal calprotectin for screening of patients

with suspected inflammatory bowel disease: diagnostic meta-analysis. *BMJ* 341, c3369 (2010).

▶26 Carpagnano GE, Barnes PJ, Geddes DM, Hodson ME, Kharitonov SA. Increased leukotriene B4 and interleukin-6 in exhaled breath condensate in cystic fibrosis. *Am. J. Respir. Crit. Care Med.* 167(8), 1109–1112 (2003).

▶27 Horak F Jr, Moeller A, Singer F *et al.* Longitudinal monitoring of pediatric cystic fibrosis lung disease using nitrite in exhaled breath condensate. *Pediatr. Pulmonol.* 42(12), 1198–1206 (2007).

▶28 Newport S, Amin N, Dozor AJ. Exhaled breath condensate pH and ammonia in cystic fibrosis and response to treatment of acute pulmonary exacerbations. *Pediatr. Pulmonol.* 44(9), 866–872 (2009).

▶29 Ojoo JC, Multennan SA, Kastelik JA, Morice AH, Redington AE. Exhaled breath condensate pH and exhaled nitric oxide in allergic asthma and in cystic fibrosis. *Thorax* 60(1), 22–26 (2005).

▶30 Bodini A, D'orazio C, Peroni DG *et al.* IL-8 and pH values in exhaled condensate after antibiotics in cystic fibrosis children. *Int. J. Immunopathol. Pharmacol.* 20(3), 467–472 (2007).

▶31 Ordonez CL, Henig NR, Mayer-Hamblett N *et al.* Inflammatory and microbiologic markers in induced sputum after intravenous antibiotics in cystic fibrosis. *Am. J. Respir. Crit. Care Med.* 168(12), 1471–1475 (2003).

▶32 Tunney MM, Field TR, Moriarty TF *et al.* Detection of anaerobic bacteria in high numbers in sputum from patients with cystic fibrosis. *Am. J. Respir. Crit. Care Med.* 177(9), 995–1001 (2008).

- **Demonstrates the presence of anaerobic bacteria in airway secretions from a large proportion of patients studied that are not detected by usual culture techniques.**

▶33 Tunney MM, Klem ER, Fodor AA *et al.* Use of culture and molecular analysis to determine the effect of antibiotic treatment on microbial community diversity and abundance during exacerbation in patients with cystic fibrosis. *Thorax* 66(7), 579–584 (2011).

34 Fothergill JL, Mowat E, Ledson MJ, Walshaw MJ, Winstanley C. Fluctuations in phenotypes and genotypes within populations of *Pseudomonas aeruginosa* in the cystic fibrosis lung during pulmonary exacerbations. *J. Med. Microbiol.* 59(Pt 4), 472–481 (2010).

▶35 Sanderson K, Wescombe L, Kirov SM, Champion A, Reid DW. Bacterial cyanogenesis occurs in the cystic fibrosis lung. *Eur. Respir. J.* 32(2), 329–333 (2008).

▶36 Schneider M, Muhlemann K, Droz S, Couzinet S, Casaulta C, Zimmerli S. Clinical characteristics associated with isolation of small-colony variants of *Staphylococcus aureus* and *Pseudomonas aeruginosa* from respiratory secretions of patients with cystic fibrosis. *J. Clin. Microbiol.* 46(5), 1832–1834 (2008).

▶37 Lamont IL, Konings AF, Reid DW. Iron acquisition by *Pseudomonas aeruginosa* in the lungs of patients with cystic fibrosis. *Biometals* 22(1), 53–60 (2009).

▶38 Raivio TL, Ujack EE, Rabin HR, Storey DG. Association between transcript levels of the *Pseudomonas aeruginosa* regA, regB, and toxA genes in sputa of cystic fibrosis patients. *Infect. Immun.* 62(8), 3506–3514 (1994).

▶39 Grimwood K, Semple RA, Rabin HR, Sokol PA, Woods DE. Elevated exoenzyme expression by *Pseudomonas aeruginosa* is correlated with exacerbations of lung disease in cystic fibrosis. *Pediatr. Pulmonol.* 15(3), 135–139 (1993).

40 Cystic Fibrosis Foundation Patient Registry (2009). Annual data report. Bethesda, MD, USA (2011).

41 Cystic fibrosis in Australia: 12th annual report from the Australian cystic fibrosis data registry. North Ryde, Sydney, Australia (2011).

▶42 Goss CH, Burns JL. Exacerbations in cystic fibrosis. 1: Epidemiology and pathogenesis. *Thorax* 62(4), 360–367 (2007).

- **Comprehensive review of the available literature on the epidemiology of acute pulmonary exacerbations in cystic fibrosis.**

▶43 Slieker MG, Van Gestel JP, Heijerman HG *et al.* Outcome of assisted ventilation for acute respiratory failure in cystic fibrosis. *Intensive Care Med.* 32(5), 754–758 (2006).

▶44 Ellaffi M, Vinsonneau C, Coste J *et al.* One-year outcome after severe pulmonary exacerbation in adults with cystic fibrosis. *Am. J. Respir. Crit. Care Med.* 171(2), 158–164 (2005).

▶45 Block JK, Vandemheen KL, Tullis E *et al.* Predictors of pulmonary exacerbations in patients with cystic fibrosis infected with multi-resistant bacteria. *Thorax* 61(11), 969–974 (2006).

46 Vandevanter DR, Yegin A, Morgan WJ *et al.* Design and powering of cystic fibrosis clinical trials using pulmonary exacerations as an efficacy endpoint. *J. Cyst. Fib.* doi: 10.1016/j.jcf.2011.07.003 (2011) (Epub ahead of print).

- **The authors use data on acute pulmonary exacerbations derived from a large cohort of cystic fibrosis patients to predict the number of patients required to adequately power an interventional study, dependent on which outcome measure is selected.**

▶47 Schechter MS, Shelton BJ, Margolis PA, Fitzsimmons SC. The association of socioeconomic status with outcomes in cystic fibrosis patients in the United States. *Am. J. Respir. Crit. Care Med.* 163(6), 1331–1337 (2001).

▶48 Wat D, Gelder C, Hibbitts S *et al.* The role of respiratory viruses in cystic fibrosis. *J. Cyst. Fib.* 7(4), 320–328 (2008).

▶49 Emerson J, Rosenfeld M, Mcnamara S, Ramsey B, Gibson RL. *Pseudomonas aeruginosa* and other predictors of mortality and morbidity in young children with cystic fibrosis. *Pediatr. Pulmonol.* 34(2), 91–100 (2002).

▶50 Stick SM, Brennan S, Murray C *et al.* Bronchiectasis in infants and preschool children diagnosed with cystic fibrosis after newborn screening. *J. Pediatr.* 155(5), 623–628 e621 (2009).

▶51 Ranganathan SC, Parsons F, Gangell C *et al.* Evolution of pulmonary inflammation and nutritional status in infants and young children with cystic fibrosis. *Thorax* 66(5), 408–413 (2011).

▶52 Boucher RC. New concepts of the pathogenesis of cystic fibrosis lung disease. *Eur. Respir. J.* 23(1), 146–158 (2004).

▶53 Wang EE, Prober CG, Manson B, Corey M, Levison H. Association of respiratory viral infections with pulmonary deterioration in patients with cystic fibrosis. *N. Engl. J. Med.* 311(26), 1653–1658 (1984).

▶54 Ramsey BW, Gore EJ, Smith AL, Cooney MK, Redding GJ, Foy H. The effect of respiratory viral infections on patients with cystic fibrosis. *Am. J. Dis. Child.* 143(6), 662–668 (1989).

▶55 Smyth AR, Smyth RL, Tong CY, Hart CA, Heaf DP. Effect of respiratory virus infections including rhinovirus on clinical status in cystic fibrosis. *Arch. Dis. Child.* 73(2), 117–120 (1995).

▶56 Oliver BG, Lim S, Wark P *et al.* Rhinovirus exposure impairs immune responses to bacterial products in human alveolar macrophages. *Thorax* 63(6), 519–525 (2008).

▶57 Van Ewijk BE, Wolfs TF, Aerts PC *et al.* RSV mediates *Pseudomonas aeruginosa* binding to cystic fibrosis and normal epithelial cells. *Pediatr. Res.* 61(4), 398–403 (2007).

▶58 France MW, Tai S, Masel PJ *et al.* The month of July: an early experience with pandemic influenza A (H1N1) in adults with cystic fibrosis. *BMC Pulm. Med.* 10, 8 (2010).

▶59 Nash EF, Whitmill R, Barker B, Rashid R, Whitehouse JL, Honeybourne D. Clinical outcomes of pandemic (H1N1) 2009

influenza (swine flu) in adults with cystic fibrosis. *Thorax* 66(3), 259 (2011).

60 Viviani L, Assael BM, Kerem E. Impact of the A (H1N1) pandemic influenza (season 2009–2010) on patients with cystic fibrosis. *J. Cyst. Fib.* 10(5), 370–376 (2011).

61 Rau MH, Hansen SK, Johansen HK *et al.* Early adaptive developments of *Pseudomonas aeruginosa* after the transition from life in the environment to persistent colonization in the airways of human cystic fibrosis hosts. *Environ. Microbiol.* 12(6), 1643–1658 (2010).

62 Kobayashi H, Kobayashi O, Kawai S. Pathogenesis and clinical manifestations of chronic colonization by *Pseudomonas aeruginosa* and its biofilms in the airway tract. *J. Infect. Chemother.* 15(3), 125–142 (2009).

63 Kobayashi H. Airway biofilms: implications for pathogenesis and therapy of respiratory tract infections. *Treat. Respir. Med.* 4(4), 241–253 (2005).

64 Aaron SD, Ramotar K, Ferris W *et al.* Adult cystic fibrosis exacerbations and new strains of *Pseudomonas aeruginosa*. *Am. J. Respir. Crit. Care Med.* 169(7), 811–815 (2004).

65 Zemanick ET, Wagner BD, Harris JK, Wagener JS, Accurso FJ, Sagel SD. Pulmonary exacerbations in cystic fibrosis with negative bacterial cultures. *Pediatr. Pulmonol.* 45(6), 569–577 (2010).

66 Worlitzsch D, Tarran R, Ulrich M *et al.* Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. *J. Clin. Invest.* 109(3), 317–325 (2002).

67 Bartlett JG. The role of anaerobic bacteria in lung abscess. *Clin. Infect. Dis.* 40(7), 923–925 (2005).

68 Torres A, Ewig S, Lode H, Carlet J. Defining, treating and preventing hospital acquired pneumonia: European perspective. *Intensive Care Med.* 35(1), 9–29 (2009).

69 Jones AM. Anaerobic bacteria in cystic fibrosis: pathogens or harmless commensals? *Thorax* 66(7), 558–559 (2011).

70 Muller FM, Seidler M. Characteristics of pathogenic fungi and antifungal therapy in cystic fibrosis. *Exp. Rev. Anti. Infect. Ther.* 8(8), 957–964 (2010).

71 Moss RB. Allergic bronchopulmonary aspergillosis and *Aspergillus* infection in cystic fibrosis. *Curr. Opin. Pulm. Med.* 16(6), 598–603 (2010).

72 Amin R, Dupuis A, Aaron SD, Ratjen F. The effect of chronic infection with *Aspergillus fumigatus* on lung function and hospitalization in patients with cystic fibrosis. *Chest* 137(1), 171–176 (2010).

73 Blondeau K, Dupont LJ, Mertens V *et al.* Gastro-oesophageal reflux and aspiration of gastric contents in adult patients with cystic fibrosis. *Gut* 57(8), 1049–1055 (2008).

74 Mohammed A, Neujahr DC. Gastroesophageal reflux disease and graft failure after lung transplantation. *Transplant. Rev. (Orlando)* 24(2), 99–103 (2010).

75 Sanduleanu S, Jonkers D, De Bruine A, Hameeteman W, Stockbrugger RW. Non-*Helicobacter pylori* bacterial flora during acid-suppressive therapy: differential findings in gastric juice and gastric mucosa. *Aliment. Pharmacol. Ther.* 15(3), 379–388 (2001).

76 Boesch RP, Acton JD. Outcomes of fundoplication in children with cystic fibrosis. *J. Pediatr. Surg.* 42(8), 1341–1344 (2007).

77 Lim WS, Baudouin SV, George RC *et al.* BTS guidelines for the management of community acquired pneumonia in adults: update 2009. *Thorax* 64(Suppl. 3), iii1–iii55 (2009).

78 Mandell LA, Wunderink RG, Anzueto A *et al.* Infectious Diseases Society of America/American Thoracic Society Consensus guidelines on the management of community – acquired pneumonia in adults. *Clin. Infect. Dis.* 44(Suppl. 2), S27–S72 (2007).

79 Rogers GB, Daniels TW, Tuck A *et al.* Studying bacteria in respiratory specimens by using conventional and molecular microbiological approaches. *BMC Pulm. Med.* 9, 14 (2009).

80 Rasmussen TB, Givskov M. Quorum-sensing inhibitors as anti-pathogenic drugs. *Int. J. Med. Microbiol.* 296(2–3), 149–161 (2006).

81 Smith AL, Fiel SB, Mayer-Hamblett N, Ramsey B, Burns JL. Susceptibility testing of *Pseudomonas aeruginosa* isolates and clinical response to parenteral antibiotic administration: lack of association in cystic fibrosis. *Chest* 123(5), 1495–1502 (2003).

82 Antibiotic consensus group. Antibiotic treatment of cystic fibrosis: report of the UK cystic fibrosis trust antibiotic working group (3rd). Cystic Fibrosis Trust (2009).

- **Comprehensive review of antibiotic treatment of cystic fibrosis airway infections based on infecting bacteria.**

83 Therapeutic guidelines: antibiotics. Expert group for respiratory infections (14). Therapeutic Guidelines Limited, Melbourne (2010).

84 Lang BJ, Aaron SD, Ferris W, Hebert PC, Macdonald NE. Multiple combination bactericidal antibiotic testing for patients with cystic fibrosis infected with multiresistant strains of *Pseudomonas aeruginosa*. *Am. J. Respir. Crit. Care Med.* 162(6), 2241–2245 (2000).

85 Aaron SD, Vandemheen KL, Ferris W *et al.* Combination antibiotic susceptibility testing to treat exacerbations of cystic fibrosis associated with multiresistant bacteria: a randomised, double-blind, controlled clinical trial. *Lancet* 366(9484), 463–471 (2005).

86 Hill D, Rose B, Pajkos A *et al.* Antibiotic susceptabilities of *Pseudomonas aeruginosa* isolates derived from patients with cystic fibrosis under aerobic, anaerobic, and biofilm conditions. *J. Clin. Microbiol.* 43(10), 5085–5090 (2005).

87 Moskowitz SM, Foster JM, Emerson J, Burns JL. Clinically feasible biofilm susceptibility assay for isolates of *Pseudomonas aeruginosa* from patients with cystic fibrosis. *J. Clin. Microbiol.* 42(5), 1915–1922 (2004).

- **Comprehensive review of the use and monitoring of aminoglycosides in patients with cystic fibrosis.**

88 Moskowitz SM, Emerson JC, Mcnamara S *et al.* Randomized trial of biofilm testing to select antibiotics for cystic fibrosis airway infection. *Pediatr. Pulmonol.* 46(2), 184–192 (2011).

89 Wientzen R, Prestidge CB, Kramer RI, Mccracken GH, Nelson JD. Acute pulmonary exacerbations in cystic fibrosis. A double-blind trial of tobramycin and placebo therapy. *Am. J. Dis. Child.* 134(12), 1134–1138 (1980).

90 Gold R, Carpenter S, Heurter H, Corey M, Levison H. Randomized trial of ceftazidime versus placebo in the management of acute respiratory exacerbations in patients with cystic fibrosis. *J. Pediatr.* 111(6 Pt 1), 907–913 (1987).

91 Smyth A, Elborn JS. Exacerbations in cystic fibrosis: 3 – Management. *Thorax* 63(2), 180–184 (2008).

- **Comprehensive review of the treatment of pulmonary exacerbations in cystic fibrosis.**

92 Emerson J, Mcnamara S, Buccat AM, Worrell K, Burns JL. Changes in cystic fibrosis sputum microbiology in the United States between 1995 and 2008. *Pediatr. Pulmonol.* 45(4), 363–370 (2010).

93 Valerius NH, Koch C, Hoiby N. Prevention of chronic *Pseudomonas aeruginosa* colonisation in cystic fibrosis by early treatment. *Lancet* 338(8769), 725–726 (1991).

94 Wiesemann HG, Steinkamp G, Ratjen F *et al.* Placebo-controlled, double-blind, randomized study of aerosolized tobramycin for early treatment of *Pseudomonas aeruginosa* colonization in cystic fibrosis. *Pediatr. Pulmonol.* 25(2), 88–92 (1998).

95 Ratjen F, Doring G, Nikolaizik WH. Effect of inhaled tobramycin on early *Pseudomonas aeruginosa* colonisation in patients with cystic fibrosis. *Lancet* 358(9286), 983–984 (2001).

96 Ratjen F, Munck A, Kho P, Angyalosi G. Treatment of early *Pseudomonas aeruginosa* infection in patients with cystic fibrosis: the ELITE trial. *Thorax* 65(4), 286–291 (2010).

97 Wainwright C, Vidmer S, Armstrong D *et al.* Effect of bronchoalveolar lavage-directed therapy on *Pseudomonas aeruginosa* infection and structural lung injury in children with cystic fibrosis. A randomized trial. *JAMA* 306(2), 163–171 (2011).

98 Courtney JM, Dunbar KE, McDowell A *et al.* Clinical outcome of *Burkholderia cepacia* complex infection in cystic fibrosis adults. *J. Cyst. Fib.* 3(2), 93–98 (2004).

99 Boussaud V, Guillemain R, Grenet D *et al.* Clinical outcome following lung transplantation in patients with cystic fibrosis colonised with *Burkholderia cepacia* complex: results from two French centres. *Thorax* 63(8), 732–737 (2008).

100 Murray S, Charbeneau J, Marshall BC, Lipuma JJ. Impact of *Burkholderia* infection on lung transplantation in cystic fibrosis. *Am. J. Respir. Crit. Care Med.* 178(4), 363–371 (2008).

101 Waters V, Yau Y, Prasad S *et al.* *Stenotrophomonas maltophilia* in cystic fibrosis: serologic response and effect on lung disease. *Am. J. Respir. Crit. Care Med.* 183(5), 635–640 (2011).

102 Sawicki GS, Rasouliyan L, Ren CL. The impact of MRSA on lung function in patients with cystic fibrosis. *Am. J. Respir. Crit. Care Med.* 179(8), 734–735; author reply 735 (2009).

103 Dasenbrook EC, Checkley W, Merlo CA, Konstan MW, Lechtzin N, Boyle MP. Association between respiratory tract methicillin-resistant *Staphylococcus aureus* and survival in cystic fibrosis. *JAMA* 303(23), 2386–2392 (2010).

104 Smyth A, Tan KH, Hyman-Taylor P *et al.* Once versus three-times daily regimens of tobramycin treatment for pulmonary exacerbations of cystic fibrosis – the TOPIC study: a randomised controlled trial. *Lancet* 365(9459), 573–578 (2005).

105 Smyth AR, Bhatt J. Once-daily versus multiple-daily dosing with intravenous aminoglycosides for cystic fibrosis. *Cochrane Database Syst. Rev.* 1, CD002009 (2010).

106 Al-Aloul M, Miller H, Alapati S, Stockton PA, Ledson MJ, Walshaw MJ. Renal impairment in cystic fibrosis patients due to repeated intravenous aminoglycoside use. *Pediatr. Pulmonol.* 39(1), 15–20 (2005).

107 Mulheran M, Degg C, Burr S, Morgan DW, Stableforth DE. Occurrence and risk of cochleotoxicity in cystic fibrosis patients receiving repeated high-dose aminoglycoside therapy. *Antimicrob. Agents Chemother.* 45(9), 2502–2509 (2001).

108 Scheenstra RJ, Rijntjes E, Tavy DL, Kingma H, Heijerman HG. Vestibulotoxicity as a consequence of systemically administered tobramycin in cystic fibrosis patients. *Acta Otolaryngol.* 129(1), 4–7 (2009).

109 Smyth A, Lewis S, Bertenshaw C, Choonara I, Mcgaw J, Watson A. Case-control study of acute renal failure in patients with cystic fibrosis in the UK. *Thorax* 63(6), 532–535 (2008).

110 Smyth AR, Prayle A. Aminoglycoside use in cystic fibrosis: therapeutic strategies and toxicity. *Curr. Opin. Pulmonary Med.* 16(6), 604–610 (2010).

111 Riethmueller J, Junge S, Schroeter TW *et al.* Continuous vs thrice-daily ceftazidime for elective intravenous antipseudomonal therapy in cystic fibrosis. *Infection* 37(5), 418–423 (2009).

112 Adeboyeku D, Jones AL, Hodson ME. Twice vs three-times daily antibiotics in the treatment of pulmonary exacerbations of cystic fibrosis. *J. Cyst. Fib.* 10(1), 25–30 (2011).

113 Smyth A, Knox A. Twice vs three times daily antibiotics in the treatment of pulmonary exacerbations of cystic fibrosis. *J. Cyst. Fib.* (2011).

114 Yang I, Kim S, Bell S. Antibiotics in COPD, bronchiectasis, and cystic fibrosis. In: *Evidence-based Respiratory Medicine.* Gibson PG (Ed.). Blackwell Publishing, Oxford, UK, 389–413 (2005).

115 Burrows JA, Nissen LM, Kirkpatrick CM, Bell SC. β-lactam allergy in adults with cystic fibrosis. *J. Cyst. Fib.* 6(4), 297–303 (2007).

116 Christenson JC, Korgenski EK, Daly JA. *In vitro* activity of meropenem, imipenem, cefepime and ceftazidime against *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *J. Antimicrob. Chemother.* 45(6), 899–901 (2000).

117 Latzin P, Fehling M, Bauernfeind A, Reinhardt D, Kappler M, Griese M. Efficacy and safety of intravenous meropenem and tobramycin versus ceftazidime and tobramycin in cystic fibrosis. *J. Cyst. Fib.* 7(2), 142–146 (2008).

118 Blumer JL, Saiman L, Konstan MW, Melnick D. The efficacy and safety of meropenem and tobramycin vs ceftazidime and tobramycin in the treatment of acute pulmonary exacerbations in patients with cystic fibrosis. *Chest* 128(4), 2336–2346 (2005).

119 Walsh TR. Clinically significant carbapenemases: an update. *Curr. Opin. Infect. Dis.* 21(4), 367–371 (2008).

120 Robinson CA, Kuhn RJ, Craigmyle J, Anstead MI, Kanga JE. Susceptibility of *Pseudomonas aeruginosa* to cefepime versus ceftazidime in patients with cystic fibrosis. *Pharmacotherapy* 21(11), 1320–1324 (2001).

121 Buonomo A, Nucera E, De Pasquale T *et al.* Tolerability of aztreonam in patients with cell-mediated allergy to α-lactams. *Ins. Arch. Allergy Immunol.* 155(2), 155–159 (2011).

122 Falagas ME, Kasiakou SK. Toxicity of polymyxins: a systematic review of the evidence from old and recent studies. *Crit. Care* 10(1), R27 (2006).

123 Falagas ME, Kastoris AC, Karageorgopoulos DE, Rafailidis PI. Fosfomycin for the treatment of infections caused by multidrug-resistant non-fermenting Gram-negative bacilli: a systematic review of microbiological, animal and clinical studies. *Ins. J. Antimicrob. Agents* 34(2), 111–120 (2009).

124 Kumon H, Ono N, Iida M, Nickel JC. Combination effect of fosfomycin and ofloxacin against *Pseudomonas aeruginosa* growing in a biofilm. *Antimicrob. Agents Chemother.* 39(5), 1038–1044 (1995).

125 Mirakhur A, Gallagher MJ, Ledson MJ, Hart CA, Walshaw MJ. Fosfomycin therapy for multiresistant *Pseudomonas aeruginosa* in cystic fibrosis. *J. Cyst. Fib.* 2(1), 19–24 (2003).

126 Dubois V, Arpin C, Dupart V *et al.* β-lactam and aminoglycoside resistance rates and mechanisms among *Pseudomonas aeruginosa* in French general practice (community and private healthcare centres). *J. Antimicrob. Chemother.* 62(2), 316–323 (2008).

127 Wolter JM, Bowler SD, Nolan PJ, Mccormack JG. Home intravenous therapy in cystic fibrosis: a prospective randomized trial examining clinical, quality of life and cost aspects. *Eur. Respir. J.* 10(4), 896–900 (1997).

128 Proesmans M, Heyns L, Moons P, Havermans T, De Boeck K. Real life evaluation of intravenous antibiotic treatment in a paediatric cystic fibrosis centre: outcome of home therapy is not inferior. *Respir. Med.* 103(2), 244–250 (2009).

129 Nazer D, Abdulhamid I, Thomas R, Pendleton S. Home versus hospital intravenous antibiotic therapy for acute pulmonary exacerbations in children with cystic fibrosis. *Pediatr. Pulmonol.* 41(8), 744–749 (2006).

130 Termoz A, Touzet S, Bourdy S *et al.* Effectiveness of home treatment for patients with cystic fibrosis: the intravenous

administration of antibiotics to treat respiratory infections. *Pediatr. Pulmonol.* 43(9), 908–915 (2008).

▶131 Collaco JM, Green DM, Cutting GR, Naughton KM, Mogayzel PJ Jr. Location and duration of treatment of cystic fibrosis respiratory exacerbations do not affect outcomes. *Am. J. Respir. Crit. Care Med.* 182(9), 1137–1143 (2010).

– **Large retrospective study demonstrating that home-based treatment may be equivalent to hospital-based treatment, and shorter courses of antibiotics may be as effective as traditional longer regimens.**

▶132 Retsch-Bogart GZ, Quittner AL, Gibson RL *et al.* Efficacy and safety of inhaled aztreonam lysine for airway pseudomonas in cystic fibrosis. *Chest* 135(5), 1223–1232 (2009).

▶133 Oermann CM, Retsch-Bogart GZ, Quittner AL *et al.* An 18-month study of the safety and efficacy of repeated courses of inhaled aztreonam lysine in cystic fibrosis. *Pediatr. Pulmonol.* 45(11), 1121–1134 (2010).

134 Remmington T, Jahnke N, Harkensee C. Oral anti-pseudomonal antibiotics for cystic fibrosis. *Cochrane Database Syst. Rev.* 3, CD005405 (2007).

▶135 Guidelines for the management of adults with hospital-acquired, ventilator-associated, and healthcare-associated pneumonia. *Am. J. Respir. Crit. Care Med.* 171(4), 388–416 (2005).

136 Elphick HE, Tan A. Single versus combination intravenous antibiotic therapy for people with cystic fibrosis. *Cochrane Database Syst. Rev.* 2, CD002007 (2005).

▶137 Flume PA, Mogayzel PJ Jr, Robinson KA *et al.* Cystic fibrosis pulmonary guidelines: treatment of pulmonary exacerbations. *Am. J. Respir. Crit. Care Med.* 180(9), 802–808 (2009).

– **Provides guidance on areas of current controversy in the treatment of pulmonary exacerbations, including combination antibiotic treatment, aminoglycoside monitoring, site and duration of treatment, and antibiotic synergy testing.**

▶138 Burrows JA, Toon M, Bell SC. Antibiotic desensitization in adults with cystic fibrosis. *Respirology* 8(3), 359–364 (2003).

139 Plummer A, Wildman M. Duration of intravenous antibiotic therapy in people with cystic fibrosis. *Cochrane Database Syst. Rev.* 1, CD006682 (2011).

▶140 Falagas ME, Avgeri SG, Matthaiou DK, Dimopoulos G, Siempos II. Short-versus long-duration antimicrobial treatment for exacerbations of chronic bronchitis: a meta-analysis. *J. Antimicrob. Chemother.* 62(3), 442–450 (2008).

▶141 Chastre J, Wolff M, Fagon JY *et al.* Comparison of 8 vs 15 days of antibiotic therapy for ventilator-associated pneumonia in adults: a randomized trial. *JAMA* 290(19), 2588–2598 (2003).

▶142 Bell SC, Bowerman AM, Nixon LE, Macdonald IA, Elborn JS, Shale DJ. Metabolic and inflammatory responses to pulmonary exacerbation in adults with cystic fibrosis. *Eur. J. Clin. Invest.* 30(6), 553–559 (2000).

▶143 Charles PG, Grayson ML. The dearth of new antibiotic development: why we should be worried and what we can do about it. *Med. J. Aust.* 181(10), 549–553 (2004).

▶144 Trials and tribulations of antibiotic development. *Lancet Infect. Dis.* 8(4), 209 (2008).

▶145 Van Westreenen M, Tiddens HA. New antimicrobial strategies in cystic fibrosis. *Paediatr. Drugs* 12(6), 343–352 (2010).

▶146 Ibrahim BM, Tsifansky MD, Yang Y, Yeo Y. Challenges and advances in the development of inhalable drug formulations for cystic fibrosis lung disease. *Exp. Opin. Drug Deliv.* 8(4), 451–466 (2011).

147 Bjarnsholt T, Jensen PO, Rasmussen TB *et al.* Garlic blocks quorum sensing and promotes rapid clearing of pulmonary *Pseudomonas aeruginosa* infections. *Microbiology* 151(Pt 12), 3873–3880 (2005).

▶148 Harjai K, Kumar R, Singh S. Garlic blocks quorum sensing and attenuates the virulence of *Pseudomonas aeruginosa*. *FEMS Immunol. Med. Microbiol.* 58(2), 161–168 (2010).

▶149 Smyth AR, Cifelli PM, Ortori CA *et al.* Garlic as an inhibitor of *Pseudomonas aeruginosa* quorum sensing in cystic fibrosis – a pilot randomized controlled trial. *Pediatr. Pulmonol.* 45(4), 356–362 (2010).

▶150 Webb JS, Thompson LS, James S *et al.* Cell death in *Pseudomonas aeruginosa* biofilm development. *J. Bacteriol.* 185(15), 4585–4592 (2003).

▶151 Reid DW, Anderson GJ, Lamont IL. Role of lung iron in determining the bacterial and host struggle in cystic fibrosis. *Am. J. Physiol. Lung Cell Mol. Physiol.* 297(5), L795–L802 (2009).

▶152 Musk DJ, Banko DA, Hergenrother PJ. Iron salts perturb biofilm formation and disrupt existing biofilms of *Pseudomonas aeruginosa*. *Chem. Biol.* 12(7), 789–796 (2005).

153 O'May CY, Sanderson K, Roddam LF, Kirov SM, Reid DW. Iron-binding compounds impair *Pseudomonas aeruginosa* biofilm formation, especially under anaerobic conditions. *J. Med. Microbiol.* 58(Pt 6), 765–773 (2009).

▶154 Musk DJ Jr, Hergenrother PJ. Chelated iron sources are inhibitors of *Pseudomonas aeruginosa* biofilms and distribute efficiently in an *in vitro* model of drug delivery to the human lung. *J. Appl. Microbiol.* 105(2), 380–388 (2008).

▶155 Braun V, Pramanik A, Gwinner T, Koberle M, Bohn E. Sideromycins: tools and antibiotics. *Biometals* 22(1), 3–13 (2009).

▶156 Dovey M, Aitken ML, Emerson J, McNamara S, Waltz DA, Gibson RL. Oral corticosteroid therapy in cystic fibrosis patients hospitalized for pulmonary exacerbation: a pilot study. *Chest* 132(4), 1212–1218 (2007).

▶157 Hester KL, Powell T, Downey DG, Elborn JS, Jarad NA. Glucocorticoids as an adjuvant treatment to intravenous antibiotics for cystic fibrosis pulmonary exacerbations: a UK Survey. *J. Cyst. Fib.* 6(4), 311–313 (2007).

▶158 Bell SC, Robinson PJ. Exacerbations in cystic fibrosis: 2. Prevention. *Thorax* 62(8), 723–732 (2007).

▶159 Walters S, Britton J, Hodson ME. Hospital care for adults with cystic fibrosis: an overview and comparison between special cystic fibrosis clinics and general clinics using a patient questionnaire. *Thorax* 49(4), 300–306 (1994).

▶160 Yankaskas JR, Marshall BC, Sufian B, Simon RH, Rodman D. Cystic fibrosis adult care: consensus conference report. *Chest* 125(Suppl. 1), S1–S39 (2004).

161 Robinson KA, Mckoy N, Saldanha I, Odelola OA. Active cycle of breathing technique for cystic fibrosis. *Cochrane Database Syst. Rev.* 11, CD007862 (2010).

▶162 Konstan MW, Wagener JS, Pasta DJ *et al.* Clinical use of dornase alfa is associated with a slower rate of FEV_1 decline in cystic fibrosis. *Pediatr. Pulmonol.* 46(6), 545–553 (2011).

163 Jones AP, Wallis C. Dornase alfa for cystic fibrosis. *Cochrane Database Syst. Rev.* 3, CD001127 (2010).

▶164 Pettit RS, Johnson CE. Airway-rehydrating agents for the treatment of cystic fibrosis: past, present, and future. *Ann. Pharmacother.* 45(1), 49–59 (2011).

▶165 Koch C, Hoiby N. Diagnosis and treatment of cystic fibrosis. *Respiration* 67(3), 239–247 (2000).

▶166 Elborn JS, Prescott RJ, Stack BH *et al.* Elective versus symptomatic antibiotic treatment in cystic fibrosis patients with chronic *Pseudomonas* infection of the lungs. *Thorax* 55(5), 355–358 (2000).

167 Ryan G, Singh M, Dwan K. Inhaled antibiotics for long-term therapy in cystic fibrosis. *Cochrane Database Syst. Rev.* 3, CD001021 (2011).

168 Smyth A, Walters S. Prophylactic antibiotics for cystic fibrosis. *Cochrane Database Syst. Rev.* 3, CD001912 (2003).

▶169 Saiman L, Marshall BC, Mayer-Hamblett N *et al.* Azithromycin in patients with cystic fibrosis chronically infected with *Pseudomonas aeruginosa*: a randomized controlled trial. *JAMA* 290(13), 1749–1756 (2003).

▶170 Kabra SK, Pawaiya R, Lodha R *et al.* Long-term daily high and low doses of azithromycin in children with cystic fibrosis: a randomized controlled trial. *J. Cyst. Fib.* 9(1), 17–23 (2010).

▶171 Fauroux B. Noninvasive ventilation in cystic fibrosis. *Exp. Rev. Respir. Med.* 4(1), 39–46 (2010).

▶172 Sood N, Paradowski LJ, Yankaskas JR. Outcomes of intensive care unit care in adults with cystic fibrosis. *Am. J. Respir. Crit. Care Med.* 163(2), 335–338 (2001).

▶173 Vedam H, Moriarty C, Torzillo PJ, McWilliam D, Bye PT. Improved outcomes of patients with cystic fibrosis admitted to the intensive care unit. *J. Cyst. Fib.* 3(1), 8–14 (2004).

▶174 Caronia CG, Silver P, Nimkoff L, Gorvoy J, Quinn C, Sagy M. Use of bilevel positive airway pressure (BIPAP) in end-stage patients with cystic fibrosis awaiting lung transplantation. *Clin. Pediatr. (Phila.)* 37(9), 555–559 (1998).

▶175 Holland AE, Denehy L, Ntoumenopoulos G, Naughton MT, Wilson JW. Non-invasive ventilation assists chest physiotherapy in adults with acute exacerbations of cystic fibrosis. *Thorax* 58(10), 880–884 (2003).

▶176 Milla CE. Nutrition and lung disease in cystic fibrosis. *Clin. Chest Med.* 28(2), 319–330 (2007).

▶177 Levy LD, Durie PR, Pencharz PB, Corey ML. Effects of long-term nutritional rehabilitation on body composition and clinical status in malnourished children and adolescents with cystic fibrosis. *J. Pediatr.* 107(2), 225–230 (1985).

▶178 Dalzell AM, Shepherd RW, Dean B, Cleghorn GJ, Holt TL, Francis PJ. Nutritional rehabilitation in cystic fibrosis: a 5 year follow-up study. *J. Pediatr. Gastroenterol. Nutr.* 15(2), 141–145 (1992).

▶179 Dodge JA, Turck D. Cystic fibrosis: nutritional consequences and management. *Best Pract. Res. Clin. Gastroenterol.* 20(3), 531–546 (2006).

ORIGINAL ARTICLE
CYSTIC FIBROSIS

Pyrosequencing reveals transient cystic fibrosis lung microbiome changes with intravenous antibiotics

Daniel J. Smith[1,2,3,4,8], Alison C. Badrick[2,3,8], Martha Zakrzewski[5], Lutz Krause[5], Scott C. Bell[1,4,6], Gregory J. Anderson[2,4] and David W. Reid[1,2,3,4,7]

Affiliations: [1]Dept of Thoracic Medicine, The Prince Charles Hospital, Brisbane, QLD, Australia. [2]Iron Metabolism Laboratory, QIMR Berghofer Medical Research Institute, Brisbane, QLD, Australia. [3]Lung, Inflammation and Infection Laboratory, QIMR Berghofer Medical Research Institute, Brisbane, QLD, Australia. [4]School of Medicine, The University of Queensland, Brisbane, QLD, Australia. [5]Bioinformatics Laboratory, QIMR Berghofer Medical Research Institute, Brisbane, QLD, Australia. [6]Queensland Children's Medical Research Institute, Brisbane, QLD, Australia. [7]Menzies Research Institute, Royal Hobart Hospital, Hobart, TAS, Australia. [8]Both authors contributed equally.

Correspondence: Daniel J. Smith, Dept of Thoracic Medicine, The Prince Charles Hospital, Rode Rd, Chermside QLD 4032, Australia. E-mail: daniel_j_smith@health.qld.gov.au

ABSTRACT Chronic airway infection in adults with cystic fibrosis (CF) is polymicrobial and the impact of intravenous antibiotics on the bacterial community composition is poorly understood. We employed culture-independent molecular techniques to explore the early effects of *i.v.* antibiotics on the CF airway microbiome.

DNA was extracted from sputum samples collected from adult subjects with CF at three time-points (before starting treatment, and at day 3 and day 8–10 of *i.v.* antibiotics) during treatment of an infective pulmonary exacerbation. Microbial community profiles were derived through analysis of bacterial-derived 16S ribosomal RNA by pyrosequencing and changes over time were compared.

59 sputum samples were collected during 24 pulmonary exacerbations from 23 subjects. Between treatment onset and day 3 there was a significant reduction in the relative abundance of *Pseudomonas* and increased microbial diversity. By day 8–10, bacterial community composition was similar to pre-treatment. Changes in community composition did not predict improvements in lung function.

The relative abundance of *Pseudomonas* falls rapidly in subjects with CF receiving *i.v.* antibiotic treatment for a pulmonary exacerbation and is accompanied by an increase in overall microbial diversity. However, this effect is not maintained beyond the first week of treatment.

@ERSpublications
Changes in the CF microbiome in response to *i.v.* antibiotics are not sustained despite ongoing antibiotic pressure http://ow.ly/wzYyg

This article has supplementary material available from erj.ersjournals.com

Received: Nov 19 2013 | Accepted after revision: April 23 2014 | First published online: July 17 2014

Support statement: This work was supported by the Royal Hobart Hospital Research Foundation (Hobart, Australia) and the National Health and Medical Research Council of Australia (Canberra, Australia). D.J. Smith is the recipient of a Postgraduate scholarship from the National Health and Medical Research Council of Australia (APP1039309). G.J. Anderson is the recipient of a Senior Research Fellowship from the National Health and Medical Research Council of Australia (APP1024230). D.W. Reid is the recipient of a Practitioner Fellowship from the National Health and Medical Research Council of Australia. S.C. Bell and D.W. Reid are recipients of Queensland Health Medical Research Fellowships (QCOS013795 and QCOS013794).

Conflict of interest: Disclosures can be found alongside the online version of this article at erj.ersjournals.com

Introduction

Despite advances in the management of cystic fibrosis (CF), chronic pulmonary infection remains responsible for most patient morbidity and mortality [1]. Culture-dependent analysis of CF airway infection reveals that *Pseudomonas aeruginosa* is the dominant bacterial pathogen in most adults with CF [2]. However, the recent application of culture-independent molecular techniques, based on the sequencing of the gene encoding bacterial 16S rRNA, have revealed a complex microbiome in the CF airway [3–9].

Chronic infection with *P. aeruginosa* is associated with an increased rate of lung function decline, increased frequency of pulmonary exacerbations, impaired quality of life and increased mortality [10, 11]. Guidelines recommend that pulmonary exacerbations are treated aggressively with a combination of at least two intravenous anti-pseudomonal antibiotics for 14 days [12, 13]. However, this practice is based on limited evidence [13].

Two studies, one prospective and one a large registry-based retrospective, have suggested that the maximum clinical and metabolic response to *i.v.* antibiotics occurs in the first week of treatment, with little additional benefit being achieved by extending treatment beyond this point [14, 15]. Unfortunately, these studies did not assess the relationship between clinical improvement and microbiological response and their findings have not been considered sufficiently robust to change clinical practice.

In this study, we utilised culture-independent molecular techniques to explore the effect on the CF airway microbiome of *i.v.* antibiotic therapy administered for the treatment of an acute pulmonary exacerbation. The focus was primarily on the impact of antibiotic therapy on microbial community composition during the first week of treatment.

Methods

Participants

23 adult subjects (aged 18–54 years) with CF, admitted to hospital for *i.v.* antibiotic treatment of an acute, infective pulmonary exacerbation were recruited from The Prince Charles Hospital (Brisbane, Australia) (n=14) and the Royal Hobart Hospital (Hobart, Australia) (n=9). Institutional human research and ethics committee approval was obtained at both sites (HREC2008:2885 and H0009813, respectively). Individuals who had undergone lung transplantation or were using systemic immunosuppression were excluded. Based on standard microbiological cultures, all patients were infected with *Pseudomonas aeruginosa* (table S1).

Spontaneously expectorated sputum samples were collected at day 1, before *i.v.* antibiotics (time-point (TP)-1), and at day 3–4 (TP-2) and day 8–10 (TP-3) following commencement of *i.v.* antibiotics.

A pulmonary exacerbation was defined clinically, based on the attending physician's assessment that the subject required *i.v.* antibiotics to treat an increase in respiratory symptoms or a decline in lung function.

Participant demographics, lung function and antibiotic treatment are outlined in table 1. Disease severity was determined based on the participants best forced expiratory volume in 1 s (FEV_1) in the 12 months prior to recruitment (FEV_1 >70% mild, 40–70% moderate and <40% severe).

Sputum collection

Sputum was expectorated directly into 10 mL of RNAlater (Life Technologies, Mulgrave, VIC, Australia) and stored for at least 24 h at 4°C to allow full penetration into the sputum sample. Sputum samples were stored at -80°C for later batch DNA extraction.

DNA extraction

A saliva-free aliquot of each frozen sputum sample was selected and manually homogenised with 500 μL lysis buffer (50 mM Tris-HCl (Sigma-Aldrich, St Louis, MO, USA)), pH 6.8, 50 mM ethylenediaminetetraacetic acid (AnalaR, PA, USA), 50 mM sucrose (AnalaR), 100 mM sodium chloride (Univar, Ingleburn, NSW, Australia) and 1% SDS (Amresco, Solon, OH, USA). The samples were incubated at 37°C for 1 h with 100 μL chicken egg lysozyme (100 $mg \cdot mL^{-1}$; Sigma-Aldrich), then at 56°C with shaking overnight with 100 μL of proteinase K (20 $mg \cdot mL^{-1}$; Promega, Alexandria, NSW, Australia) and 75 μL of 10% SDS. If samples were not completely digested, overnight incubation with additional proteinase was repeated until complete digestion was achieved. DNA was extracted and purified using PureLink Genomic DNA Mini Kit (Life Technologies) and eluted in 50 μL kit elution buffer. Purified genomic DNA was analysed for quality (A_{260}/A_{280} ratio of 1.8–2.0) and concentration using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Denver, CO, USA) and samples were diluted to 20 $ng \cdot \mu L^{-1}$ prior to DNA amplification and sequencing.

DOI: 10.1183/09031936.00203013

TABLE 1 Participant demographics, cystic fibrosis genotypes and antibiotic treatment

Sex	
Male	17
Female	6
Age years	27 (18–54)
BMI kg·m^{-2}	20 (17–26)
FEV$_1$ L	1.7 (0.9–4.2)
FEV$_1$ % predicted	50 (21–97)
Absolute improvement in FEV$_1$ L	0.2 (0–1.2)
Relative improvement in FEV$_1$ %	16 (0–76)
Genotype	
p.F508del homozygote	12
p.F508del heterozygote	8
G551D heterozygote	1
Other/other	2
Intravenous antibiotic treatment[#]	
β-lactam + aminoglycoside[¶]	18
Dual β-lactams	2
Dual β-lactams + aminoglycoside	1
Dual β-lactams + colistin	1
β-lactam + monobactam + aminoglycoside	1
Azithromycin[#,+]	18

Data are presented as n or median (range). Individual participant data are available in tables S1 and S2. BMI: body mass index; FEV$_1$: forced expiratory volume in 1 s. [#]: maintenance usage of oral azithromycin, dosage either 250 mg daily or 500 mg on alternate days; [¶]: nebulised aminoglycoside was used in one subject; [+]: data were unavailable for one subject.

DNA amplification, sequencing and statistical analysis

Sequencing was performed by Research and Testing Laboratories (Lubbock, TX, USA) applying Molecular Research DNA protocols (Shallowater, TX, USA). The 16S rRNA gene was amplified using primers 939F (5′-TTGACGGGGGCCCGCAC-3′) and 1492R (5′-TACCTTGTTACGACTT-3′) and products were sequenced using bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) as described previously [16]. 16S rDNA amplicon sequences were processed with QIIME 1.5 [17]. Sequences with ambiguous base calls ("quality score" <25 and $\geqslant 6$ homopolymers) were discarded. Forward and reverse primer sequences were removed allowing one mismatch. Chimeras were removed with ChimeraSlayer [18] using default parameters. Samples with <500 sequence reads were excluded. 16S rDNA sequences were clustered into operational taxonomic units (OTUs) using UCLUST v5.2.32 [19], employing an identity threshold of 97%. RDP Classifier v2.2 was retrained on the database Greengenes version 13.8 and used for the taxonomic assignment of representative sequences of each OTU with a confidence threshold of 0.6 [20]. Genera were categorised as aerobic or anaerobic, as described previously [21]. Data-mining, statistical analysis and data visualisation were carried out using the Calypso software (bioinfo.qimr.edu.au/calypso) and Krona [22].

Groups were compared using paired t-tests: TP-1 *versus* TP-2; TP-1 *versus* TP-3; and TP2 *versus* TP-3. Analysis was limited to subjects for whom samples at both compared time-points were available. P-values were adjusted for multiple testing using the False Discovery Rate (FDR). Microbial community diversity was assessed by Shannon index (OTU level). Canonical Correlation Analysis (CCA) and ANOSIM were performed on the sputum microbiome at TP-1 using the relative genera abundance (number of 16S sequences assigned to each genus divided by the total number of sequences obtained for each sample). Associations between clinical variables (age and FEV$_1$) and the relative abundance of each genus at TP-1 or TP-3 were determined by the Pearson product-moment correlation coefficient. Analogously, Pearson correlation was utilised to infer associations between the relative abundance of genera, age and relative improvement in FEV$_1$ between early and late treatment. For the purpose of determining the differences between the relative abundances of bacterial groups at the genus level, Shannon diversity and FEV$_1$ at TP-1 and TP-3 were calculated, and then evaluated for relationships by means of Pearson correlation.

DOI: 10.1183/09031936.00203013

Real-time PCR quantification of P. aeruginosa

In 18 sputum samples from nine subjects, *P. aeruginosa* concentration was determined by real-time quantitative PCR using previously established methodologies [23]. Pearson's correlation was used to explore the relationship between *P. aeruginosa* load and relative abundance of *Pseudomonas* at the genus level.

Results

Sample quality

59 sputum samples were collected during 24 pulmonary exacerbations from 23 subjects. Sequencing yielded 420 145 reads with an average read length of 457 base-pairs. After quality control and chimera detection, 57 samples (18 for TP-1, 18 for TP-2 and 21 for TP-3) and 336 973 high-quality sequencing reads remained, with a median of 5478 sequences per sample and a range from 1468 to14 164.

Sputum microbiota before antibiotic treatment: TP-1

18 sputum specimens were analysed at TP-1 and showed a complex microbiota, with a median (range) of 113 (23–210) OTUs per sample. A total of 100 different genera were observed (median (range) 13 (2–39) per sample) (fig. 1). *Pseudomonas* was the dominant genus in 94% (17 out of 18) of samples, with a mean (range) relative abundance of 78.5% (41.9–99.6%). In one subject, *Fusobacterium* was the dominant genus (49% of 16S sequences), followed by *Pseudomonas* (41.9% of sequences). *Streptococcus* was the second most abundant genus in 13 sputum samples, with a mean relative abundance of 7.3%. The most prevalent anaerobic genera were *Prevotella* (mean (range) relative abundance 2.7%, (0–13.8%)) and *Veillonella* (mean (range) relative abundance 1.2% (0–5.9%)), which were present in 72% and 78% of the subjects, respectively. Other pathogenic genera were recovered infrequently, including *Actinomyces* (44% of samples), *Staphylococcus* (17% of samples) and *Haemophilus* (11% of samples). The mean percentage of sequences that could not be assigned to any known genus was 3% (table S3). Statistical analysis of the taxonomic profiles at TP-1 did not identify a relationship between global microbial community composition (rank genus) and age (CCA: p=0.55; ANOSIM: p=0.59) or FEV_1 (CCA: p=0.99; ANOSIM: p=0.7).

The relative abundance of *Pseudomonas* showed a strong negative correlation with microbial diversity (Shannon index; r= -0.81, p<0.01, Pearson correlation). Conversely, the relative abundance of *Streptococcus* (r=0.63, p<0.01) and *Prevotella* (r=0.63, p<0.01) were positively correlated with community diversity (fig. S1). We did not observe an association between microbial diversity and subject age (p=0.29) or lung function (FEV_1; p=0.3).

Effects of antibiotic treatment on the airway microbiome

There was a significant reduction in the relative abundance of *Pseudomonas* during the first 72 h of antibiotic treatment (p<0.005; FDR=0.02, paired t-test TP-1 *versus* TP-2). The mean relative abundance of *Pseudomonas* decreased from 78.5% of 16S sequences before antibiotic treatment to 47% after 72 h of treatment. The reduction in *Pseudomonas* was accompanied by a significant increase in overall microbial diversity (Shannon index paired t-test p=0.012) and a trend towards an increase in the relative abundance

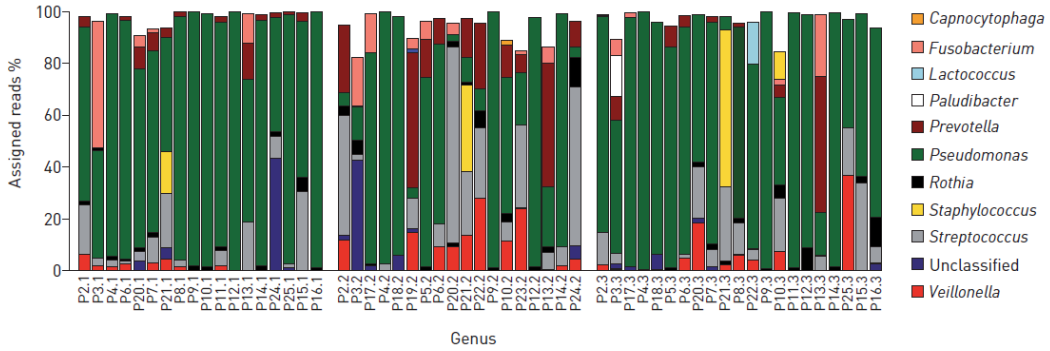


FIGURE 1 Taxonomic profile of all sputum samples segregated by time-point. Only genera which were present in at least one sample at a relative abundance of ⩾10% are presented.

DOI: 10.1183/09031936.00203013

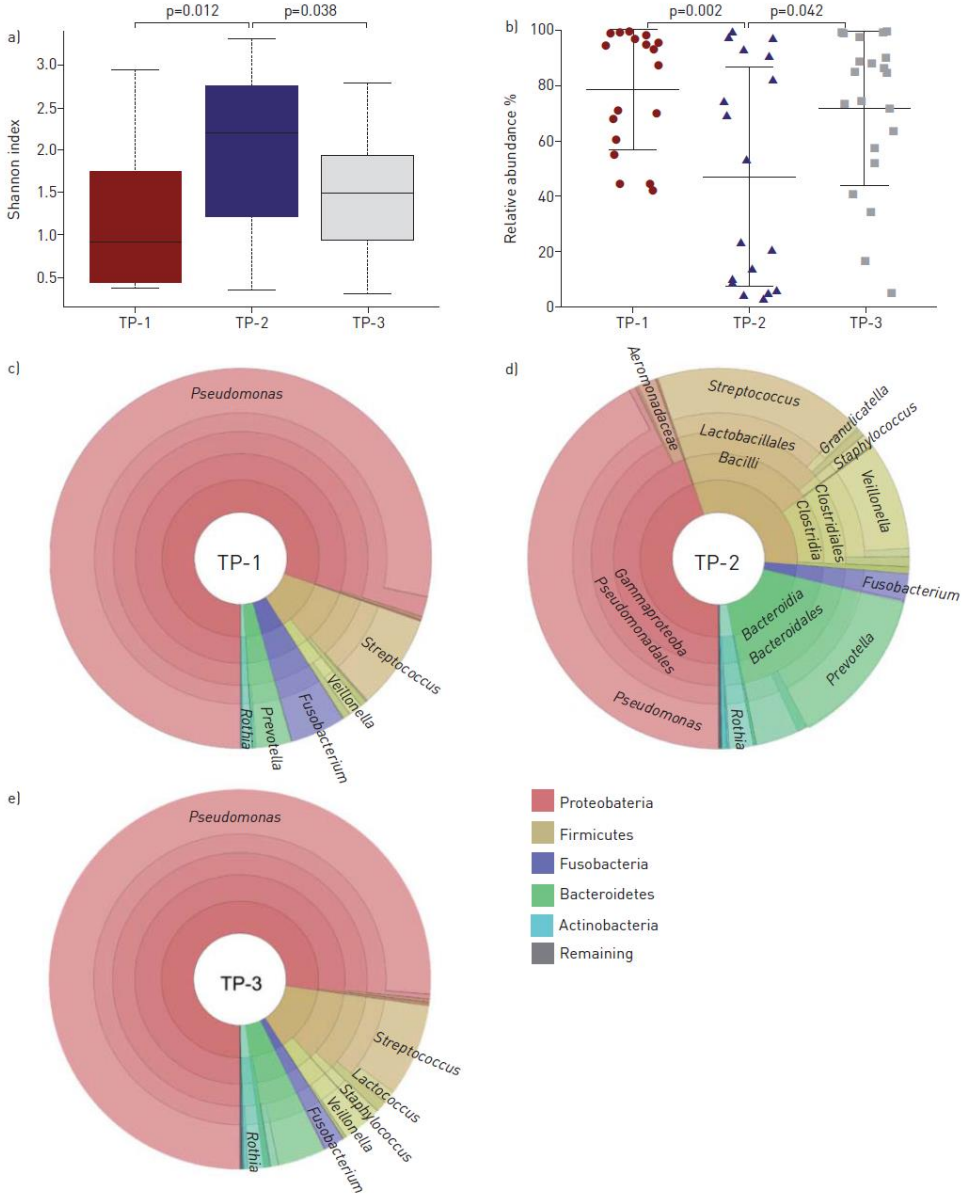


FIGURE 2 Changes in cystic fibrosis sputum microbiota between time-point (TP)-1, TP-2 and TP-3. a) Community diversity assessed by Shannon index (OTU level) demonstrating changes in overall microbial diversity. b) Changes in the relative abundance of *Pseudomonas*. c–e) Circle plots demonstrating changes in global community structure at c) TP-1, d) TP-2 and e) TP-3. Each ring represents a taxonomic rank (phylum, class, order, family and genus from inner to outer circle, respectively).

DOI: 10.1183/09031936.00203013

of anaerobes, which was mainly driven by increases in the abundance of *Prevotella* (TP-1 2.7% of 16S sequences; TP-2 12.2%; p=0.06; FDR=0.13) and *Veillonella* (TP-1 1.2%; TP-2 6.8%; p<0.02; FDR=0.07) (fig. 2).

At TP-3, overall microbial diversity (Shannon index) and *Pseudomonas* relative abundance were similar to TP-1, suggesting a return to pre-treatment community composition (fig. 2). The change in overall microbial diversity correlated negatively with the change in abundance of *Pseudomonas* (r= -0.7, p<0.01) and positively with change in abundance of *Streptococcus* (r=0.6, p<0.05) (fig. S2).

A principal coordinates analysis demonstrated samples from TP-1 and TP-3 clustered together; however, TP-2 formed a distant separate cluster (fig. S3c). This clustering was significant; comparison of intra- and intergroup Jarrard distances of TP-1 *versus* TP-3 were not significant (p=0.75), but were significant between TP-1 *versus* TP-2 and TP-2 *versus* TP-3 (p<0.01).

We did not observe any significant associations between the changes in abundance of individual genera and improvement in FEV_1 between TP-1 and TP-3. A negative relationship between relative abundance of *Pseudomonas* and FEV_1 was observed at TP-3, but did not reach statistical significance (r= -0.46, p=0.07).

In the nine subjects for whom *P. aeruginosa* quantification was performed, there was a weak, positive correlation between *P. aeruginosa* load and the relative abundance of *Pseudomonas* at the genus level (r^2=0.24, p=0.04) (fig. S4).

Sub-group analysis based on the relative abundance of P. aeruginosa

Clustering of OTUs based on the relative abundance of *Pseudomonas* spp. revealed three distinct groups: low abundance of *Pseudomonas* spp. (<40%) with a complex community structure and presence of various other bacterial genera; medium abundance of *Pseudomonas* spp. (40 to <75%); and high abundance of *Pseudomonas* spp. (⩾75%) (fig. S3b).

Samples from subjects with severe disease tended to cluster in the high and medium categories, whereas samples from subjects with mild-to-moderate disease clustered in the low category (fig. S3c). Antibiotic treatment rarely resulted in a change in a subject's category at the end of treatment, and changes in the relative abundance of *Pseudomonas* in response to treatment were not associated with an improvement in lung function (fig. S2).

Non-*Pseudomonas* genera were categorised as obligate anaerobes or aerobes/facultative anaerobes (fig. 3). By TP-2 the median relative abundance of aerobic bacteria increased (TP-1: 4.5% of 16S sequences; TP-2: 11.7% of 16S sequences; p=0.03, FDR=0.049) and a similar trend was observed in the abundance of anaerobic bacteria (TP-1: 3.4%; TP-2: 22.6%; p=0.1). A reversal in these trends was seen between TP-2 and TP-3 (aerobes TP-3: median 9.5%, paired t-test between TP-2 and TP-3 p=0.44; anaerobes TP-3: median: 2.4%, p=0.048, FDR=0.1) and at TP-3, the sputum community composition once again resembled TP-1 (fig. 3).

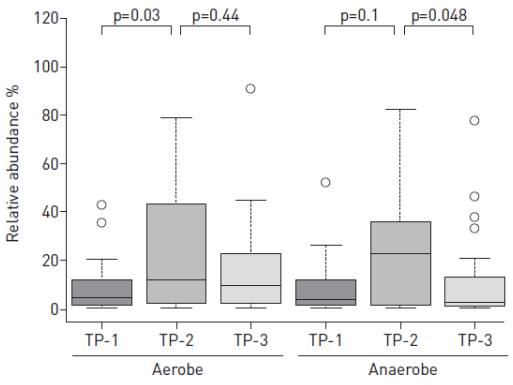


FIGURE 3 Relative abundance (median) of aerobic and anaerobic genera in cystic fibrosis sputum samples after exclusion of reads assigned to *Pseudomonas*. There was a significant increase in the relative abundance of aerobic bacteria between time-point (TP)-1 and TP-2, with a similar trend seen in anaerobic bacteria, followed by a significant decrease in anaerobic bacteria between TP-2 and TP-3. The p-values were calculated using a paired t-test. Changes in the relative abundance of aerobic and anaerobic genera in response to treatment in individual subjects are presented in figure S6.

DOI: 10.1183/09031936.00203013

Discussion

In this study, we demonstrate a significant perturbation of the airway microbiome over the first 72 h of *i.v.* antibiotic treatment for pulmonary exacerbations in adult subjects with CF. This perturbation was characterised by a reduction in the dominance of *Pseudomonas* and an accompanying increase in microbial diversity. These changes were, however, short-lived, with bacterial community composition resembling that of the initial profile after 1 week of antibiotic treatment. These novel findings suggest that disturbance of the bacterial composition of the CF airway in response to *i.v.* antibiotics is transient, and potentially challenge current antibiotic management strategies for CF pulmonary exacerbations.

Evidence in the literature to support the application of culture-independent techniques to examine the effect of antibiotic therapy on the CF airway microbiome remains limited. TUNNEY *et al.* [9] have demonstrated that despite total bacterial numbers being reduced in response to antibiotics (culture-based assessment), there was relative stability in overall community composition as assessed by terminal restriction fragment length polymorphisms analysis when sputum samples were collected from subjects with CF before and after treatment of a pulmonary exacerbation. More recently, DANIELS *et al.* [24] examined the impact of antibiotics on CF sputum microbial diversity in a group of adult CF subjects. Sputum samples collected following the initial 72 h of antibiotic therapy were compared with samples collected during a period of clinical stability prior to the exacerbation and after 10–14 days of treatment. In comparison to samples collected at 72 h, samples collected at 10–14 days demonstrated an increase in relative abundance of *Pseudomonas* species compared with non-pseudomonads accompanied by a reduction in community diversity [24]. The authors concluded that antibiotic therapy was exerting a significantly greater effect on bacterial species other than *Pseudomonas*, culminating in the dominance of *P. aeruginosa*.

By examining microbial diversity at the onset of the exacerbation, prior to the administration of antibiotics, we have advanced the findings of DANIELS *et al.* [24] and shown that bacterial communities appear most susceptible to *i.v.* antibiotics at the beginning of antibiotic therapy. In addition to performing sampling prior to the onset of antibiotics, a number of important differences between the current study and that of DANIELS *et al.* [24] should be highlighted. In the earlier study, subjects were treated with a range of oral, inhaled and *i.v.* antibiotics which may have affected the ability to pick up consistent changes in community composition, whereas the subjects reported here were consistently treated with conventional *i.v.* antibiotic combinations. Additionally, sample processing and sequencing techniques differed between the two studies, most notably the samples in the study DANIELS *et al.* [24] were treated with propidium monoazide (PMA) to cross-link DNA from non-viable bacteria, a technique we did not employ. Nevertheless, both studies confirm that community composition remains essentially unchanged after at least 1 week of *i.v.* antibiotic therapy, which, given the early alterations in the microbiome that we observed, suggests that current treatment practices may need to be revised [25, 26].

The reduction in relative abundance of *Pseudomonas* from day 1 to days 3–4 suggests initial preferential killing of *Pseudomonas* in response to *i.v.* antibiotics. Recrudescence of *P. aeruginosa* infection under antibiotic pressure after this early response may occur as a result of increased replication of an inherently antibiotic resistant sub-population, or through population-wide adaptive mechanisms involving the upregulation of antibiotic resistance genes, which may occur rapidly in the treatment course [27, 28]. Whilst alternative explanations for the clinical improvements (reduction in respiratory symptoms and subjective wellbeing) in subjects beyond the first few days of treatment should also be considered, including the impact of adjunctive therapies, such as inhaled mucolytics, airway clearance techniques, rehydration and nutritional support [14, 29, 30], our findings challenge the convention of prescribing the same *i.v.* antibiotic combination for more than a few days at a time. A potential new strategy may involve the rapid cycling of different antibiotic regimes during treatment, but this would require investigation in large-scale, adequately powered randomised and blinded clinical trials.

To explore the effect of antibiotics on bacterial species other than *P. aeruginosa*, we examined microbial community changes with *Pseudomonas* reads excluded. Contrary to the earlier study by DANIELS *et al.* [24] the relative abundance of both aerobes and anaerobes increased in the early stages of treatment, suggesting antibiotic therapy was having a lesser impact on the other bacterial species present compared to *Pseudomonas*. The effects of *i.v.* antibiotics in adults not infected with *P. aeruginosa* who are experiencing an acute exacerbation warrants further investigation, particularly as these individuals represent an increasing group of subjects transitioning from paediatric to adult care [31].

Greater sputum microbial diversity in our study subjects was positively associated with the abundance of *Streptococcus*, accompanied by a reduction in the relative abundance of *Pseudomonas*. These findings are consistent with a previous cross-sectional study in which microbial profiles of stable CF outpatients and inpatients were compared [32]. In this earlier report, three distinct sub-groups of CF subjects were identified by the relative abundance of *Streptococcus* and *Pseudomonas* in sputum. Importantly, outpatients

DOI: 10.1183/09031936.00203013

with high *Streptococcus* and low *Pseudomonas* abundance had greater lung function stability over time. In our study, sputum samples with a high relative abundance of *Pseudomonas* and low community diversity formed a cluster, predominantly in subjects with severe lung disease. However, in contrast to other studies, we were unable to confirm a reduction in microbial diversity with increasing age and severity of lung disease in the CF subjects we studied [25, 33].

One potential limitation of our study is that PMA was not used to exclude DNA from non-viable bacterial cells in the sputum samples prior to analysis [34]. The use of PMA has been advocated due to appropriate theoretical concerns that molecular-based techniques may identify both viable and non-viable bacteria and limit the ability to detect changes in bacterial number in antibiotic treatment responses studies [35]. However, the reduction in the relative abundance of *Pseudomonas* we observed at day 3 would not be consistent with the inclusion of non-viable organisms [34]. The use of PMA remains a topic of debate and the method carries its own potential drawbacks, including a limited ability to expediently penetrate purulent, non-homogenised sputum prior to the death of resident organisms deep within the sputum sample. Furthermore, the extended processing time involved may result in less hardy bacteria (*e.g.* anaerobes) preferentially dying *ex vivo*, while more robust bacteria proliferate and skew the true bacterial composition, even if samples are maintained at 4°C [9, 36].

Our conclusions are based on changes in relative abundance and not quantitative bacterial load. We have previously performed enumeration of *P. aeruginosa* number by real-time quantitative PCR and demonstrated heterogeneous changes in bacterial numbers in response to antibiotics [23]. The findings for some subjects in this earlier work were consistent with the current study, with an early reduction in *P. aeruginosa* numbers being followed by a recrudescence by the end of the first week of treatment [23]. Quantification of *P. aeruginosa* by real-time quantitative PCR in nine subjects from the current study revealed a positive correlation between *P. aeruginosa* load and the relative abundance of *Pseudomonas* at the genus level (fig. S4), and supports conjecture that changes in community composition in the CF lung reflect changes in *P. aeruginosa* concentration; however, this relationship was weak. Matching changes in community structure with enumeration of individual bacterial species is complex and changes in individual species may not be equivalent to changes at the level of the genus. Future advances in pyrosequencing technology may allow for identification and enumeration of bacteria at the species level, which will substantially advance understanding of the dynamics of the lung microbiome.

Approximately 3% of all obtained sequences in our study could not be assigned to a genus, which is consistent with the findings of other studies [37]. The success of read assignment is dependent on read length, primer pairs and the particular reference database applied to the analysis [38]. To date, there no universal method of analysis is agreed upon, which further confounds direct comparisons of the human microbiome between studies.

In summary, we have demonstrated for the first time that the relative abundance of *P. aeruginosa* falls rapidly in subjects with CF receiving *i.v.* antibiotics for pulmonary exacerbations, and that this is accompanied by an increase in microbial diversity. This effect was not maintained beyond the first week of treatment. These findings have implications for how *i.v.* antibiotic treatment should be employed for exacerbations and for how long. Future clinical trials should consider the impact of *i.v.* antibiotics on the whole lung microbiome in CF, how changes in microbial community composition relate to reductions in absolute bacterial counts and, in turn, how these parameters relate to the clinical response to treatment.

Acknowledgements

We would like to thank to Dana Willner (Australian Centre for Ecogenomics, University of Queensland, Brisbane, Australia and Diamantina Institute, University of Queensland, Brisbane, Australia) for help and advice with the initial optimisation of DNA extraction protocols.

References

1 Cystic Fibrosis Foundation. Patient Registry: Annual Data Report 2010. Bethesda, MD, USA.
2 Williams HD, Davies JC. Basic science for the chest physician: *Pseudomonas aeruginosa* and the cystic fibrosis airway. *Thorax* 2012; 67: 465–467.
3 Rogers GB, Daniels TW, Tuck A, *et al.* Studying bacteria in respiratory specimens by using conventional and molecular microbiological approaches. *BMC Pulm Med* 2009; 9: 14.
4 de Vrankrijker AM, Wolfs TF, van der Ent CK. Challenging and emerging pathogens in cystic fibrosis. *Paediatr Respir Rev* 2010; 11: 246–254.
5 Harrison F. Microbial ecology of the cystic fibrosis lung. *Microbiology* 2007; 153: 917–923.
6 Sibley CD, Grinwis ME, Field TR, *et al.* Culture enriched molecular profiling of the cystic fibrosis airway microbiome. *PLoS One* 2011; 6: e22702.
7 Rogers GB, Carroll MP, Serisier DJ, *et al.* Characterization of bacterial community diversity in cystic fibrosis lung infections by use of 16s ribosomal DNA terminal restriction fragment length polymorphism profiling. *J Clin Microbiol* 2004; 42: 5176–5183.

8 VanDevanter DR, LiPuma JJ. Microbial diversity in the cystic fibrosis airways: where is thy sting? *Future Microbiol* 2012; 7: 801–803.
9 Tunney MM, Klem ER, Fodor AA, *et al*. Use of culture and molecular analysis to determine the effect of antibiotic treatment on microbial community diversity and abundance during exacerbation in patients with cystic fibrosis. *Thorax* 2011; 66: 579–584.
10 Cohen-Cymberknoh M, Shoseyov D, Kerem E. Managing cystic fibrosis: strategies that increase life expectancy and improve quality of life. *Am J Respir Crit Care Med* 2011; 183: 1463–1471.
11 Kerem E, Viviani L, Zolin A, *et al*. Factors associated with FEV1 decline in cystic fibrosis: analysis of the data of the ECFS Patient Registry. *Eur Respir J* 2014; 43: 125–133.
12 Doring G, Flume P, Heijerman H, *et al*. Treatment of lung infection in patients with cystic fibrosis: current and future strategies. *J Cyst Fibros* 2012; 11: 461–479.
13 Plummer A, Wildman M. Duration of intravenous antibiotic therapy in people with cystic fibrosis. *Cochrane Database Syst Rev* 2011; 1: CD006682.
14 Bell SC, Bowerman AM, Nixon LE, *et al*. Metabolic and inflammatory responses to pulmonary exacerbation in adults with cystic fibrosis. *Eur J Clin Invest* 2000; 30: 553–559.
15 Collaco JM, Green DM, Cutting GR, *et al*. Location and duration of treatment of cystic fibrosis respiratory exacerbations do not affect outcomes. *Am J Respir Crit Care Med* 2010; 182: 1137–1143.
16 Dowd SE, Sun Y, Wolcott RD, *et al*. Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) for microbiome studies: bacterial diversity in the ileum of newly weaned *Salmonella*-infected pigs. *Foodborne Pathog Dis* 2008; 5: 459–472.
17 Caporaso JG, Kuczynski J, Stombaugh J, *et al*. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 2010; 7: 335–336.
18 Haas BJ, Gevers D, Earl AM, *et al*. Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. *Genome Res* 2011; 21: 494–504.
19 Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 2010; 26: 2460–2461.
20 DeSantis TZ, Hugenholtz P, Larsen N, *et al*. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* 2006; 72: 5069–5072.
21 Rogers GB, Cuthbertson L, Hoffman LR, *et al*. Reducing bias in bacterial community analysis of lower respiratory infections. *ISME J* 2013; 7: 697–706.
22 Ondov BD, Bergman NH, Phillippy AM. Interactive metagenomic visualization in a Web browser. *BMC Bioinformatics* 2011; 12: 385.
23 Reid DW, Latham R, Lamont IL, *et al*. Molecular analysis of changes in *Pseudomonas aeruginosa* load during treatment of a pulmonary exacerbation in cystic fibrosis. *J Cyst Fibros* 2013; 12: 688–699.
24 Daniels TW, Rogers GB, Stressmann FA, *et al*. Impact of antibiotic treatment for pulmonary exacerbations on bacterial diversity in cystic fibrosis. *J Cyst Fibros* 2013; 12: 22–28.
25 Zhao J, Schloss PD, Kalikin LM, *et al*. Decade-long bacterial community dynamics in cystic fibrosis airways. *Proc Natl Acad Sci USA* 2012; 109: 5809–5814.
26 Stressmann FA, Rogers GB, Marsh P, *et al*. Does bacterial density in cystic fibrosis sputum increase prior to pulmonary exacerbation? *J Cyst Fibros* 2011; 10: 357–365.
27 Breidenstein EB, de la Fuente-Nunez C, Hancock RE. *Pseudomonas aeruginosa*: all roads lead to resistance. *Trends Microbiol* 2011; 19: 419–426.
28 Fothergill JL, Mowat E, Ledson MJ, *et al*. Fluctuations in phenotypes and genotypes within populations of *Pseudomonas aeruginosa* in the cystic fibrosis lung during pulmonary exacerbations. *J Med Microbiol* 2010; 59: 472–481.
29 Flume PA, Mogayzel PJ Jr, Robinson KA, *et al*. Cystic fibrosis pulmonary guidelines: treatment of pulmonary exacerbations. *Am J Respir Crit Care Med* 2009; 180: 802–808.
30 Flume PA, Robinson KA, O'Sullivan BP, *et al*. Cystic fibrosis pulmonary guidelines: airway clearance therapies. *Respir Care* 2009; 54: 522–537.
31 Lipuma JJ. The changing microbial epidemiology in cystic fibrosis. *Clin Microbiol Rev* 2010; 23: 299–323.
32 Filkins LM, Hampton TH, Gifford AH, *et al*. Prevalence of streptococci and increased polymicrobial diversity associated with cystic fibrosis patient stability. *J Bacteriol* 2012; 194: 4709–4717.
33 Cox MJ, Allgaier M, Taylor B, *et al*. Airway microbiota and pathogen abundance in age-stratified cystic fibrosis patients. *PLoS One* 2010; 5: e11044.
34 Rogers GB, Stressmann FA, Koller G, *et al*. Assessing the diagnostic importance of nonviable bacterial cells in respiratory infections. *Diagn Microbiol Infect Dis* 2008; 62: 133–141.
35 Rogers GB, Marsh P, Stressmann AF, *et al*. The exclusion of dead bacterial cells is essential for accurate molecular analysis of clinical samples. *Clin Microbiol Infect* 2010; 16: 1656–1658.
36 Tunney MM, Field TR, Moriarty TF, *et al*. Detection of anaerobic bacteria in high numbers in sputum from patients with cystic fibrosis. *Am J Respir Crit Care Med* 2008; 177: 995–1001.
37 Erb-Downward JR, Thompson DL, Han MK, *et al*. Analysis of the lung microbiome in the "healthy" smoker and in COPD. *PLoS One* 2011; 6: e16384.
38 Werner JJ, Koren O, Hugenholtz P, *et al*. Impact of training sets on classification of high-throughput bacterial 16s rRNA gene surveys. *ISME J* 2012; 6: 94–103.

DOI: 10.1183/09031936.00203013

OPEN ACCESS Freely available online

PLOS ONE

Reduced Mucosal Associated Invariant T-Cells Are Associated with Increased Disease Severity and *Pseudomonas aeruginosa* Infection in Cystic Fibrosis

Daniel J. Smith[1,2,4]*, Geoffrey R. Hill[3], Scott C. Bell[1,2,5], David W. Reid[1,2,4]

1 Department of Thoracic Medicine, The Prince Charles Hospital, Brisbane, Queensland, Australia, **2** School of Medicine, University of Queensland, Brisbane, Queensland, Australia, **3** Bone Marrow Transplant Laboratory, QIMR Berghofer Medical Research Institute, Brisbane, Queensland, Australia, **4** Infection and Inflammation Laboratory, QIMR Berghofer Medical Research Institute, Brisbane, Queensland, Australia, **5** Queensland Children's Medical Research Institute, Royal Children's Hospital, Brisbane, Queensland, Australia

Abstract

Background: Primary defects in host immune responses have been hypothesised to contribute towards an inability of subjects with cystic fibrosis (CF) to effectively clear pulmonary infections. Innate T-lymphocytes provide rapid pathogen-specific responses prior to the development of classical MHC class I and II restricted T-cell responses and are essential to the initial control of pulmonary infection. We aimed to examine the relationship between peripheral blood lymphocyte phenotype and clinical outcomes in adults with CF.

Methods: We studied 41 subjects with CF and 22, age matched, non-smoking healthy control subjects. Lymphocytes were extracted from peripheral blood samples and phenotyped by flow-cytometry. Lymphocyte phenotype was correlated with sputum microbiology and clinical parameters.

Results: In comparison to healthy control subjects, mucosal associated invariant T (MAIT)-lymphocytes were significantly reduced in the peripheral blood of subjects with CF (1.1% versus 2.0% of T-lymphocytes, $P = 0.002$). MAIT cell concentration was lowest in CF subjects infected with *P. aeruginosa* and in subjects receiving treatment for a pulmonary exacerbation. Furthermore a reduced MAIT cell concentration correlated with severity of lung disease.

Conclusion: Reduced numbers of MAIT cells in subjects with CF were associated with *P. aeruginosa* pulmonary infection, pulmonary exacerbations and more severe lung disease. These findings provide the impetus for future studies examining the utility of MAIT cells in immunotherapies and vaccine development. Longitudinal studies of MAIT cells as biomarkers of CF pulmonary infection are awaited.

Citation: Smith DJ, Hill GR, Bell SC, Reid DW (2014) Reduced Mucosal Associated Invariant T-Cells Are Associated with Increased Disease Severity and *Pseudomonas aeruginosa* Infection in Cystic Fibrosis. PLoS ONE 9(10): e109891. doi:10.1371/journal.pone.0109891

Editor: Johan K. Sandberg, Karolinska Institutet, Sweden

Received May 18, 2014; **Accepted** September 10, 2014; **Published** October 8, 2014

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

Funding: Internal funding was received from QIMR Berghofer Medical Research Institute. The institute had no role in decision to publish the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* Email: daniel.smith@health.qld.gov.au

Introduction

Cystic fibrosis (CF) pulmonary disease is typified by a vicious cycle of bacterial infection and exuberant, but ineffective host immune response [1]. The inability of the intense inflammatory response to clear infection has led to speculation that intrinsic immune defects may contribute to the persistence of pathogens in CF [2]. At the level of the airway lumen, the cellular immune response is dominated by activated neutrophils. However, in contrast, airway epithelial biopsies demonstrate a profound T lymphocyte (T-cell) infiltrate, supporting an important role for adaptive immune responses in the orchestration of a sustained inflammatory response [3].

To date, studies of peripheral, adaptive immune responses in CF have largely focused on the classic dichotomy of T-helper (Th)-1 and Th-2 responses [4]. These early studies suggested a skew towards a Th2 in most CF subjects with *P. aeruginosa* infection, which resulted in increased pulmonary inflammation and disease progression [4–6]. The activation of the "classical" adaptive immune response involves antigen recognition, followed by T-cell recruitment and clonal expansion at the site of infection. Consequently, there is a time lag between the host's recognition of the presence of a pathogen and the development of an effective, adaptive immune response. In recent times, an increasing number of unconventional "innate" T-cell populations have been described (including γ/δ, semi-invariant natural killer (iNKT) and M3-restricted T-cells), which are capable of mounting a more immediate response to pathogens than was previously thought possible. Mucosal associated invariant T (MAIT) cells are a recently described sub-class of innate T-cells, which can be differentiated from other T-cells by the presence of an evolutionary conserved T-cell receptor (TCR)

(Vα7.2−Jα33). MAIT cells recognise bacterial and fungal metabolites presented on the major histocompatibility complex (MHC) related protein-1 (MR1) (including the common CF pathogens *Pseudomonas aeruginosa* and *Staphylococcus aureus*), but not viruses [7,8]. These "innate" T-cell populations provide rapid pathogen-specific responses prior to the development of classical MHC class I and II restricted T-cell responses and importantly may also provide a sustained cytokine response in chronic infection [9,10].

To date, there is limited knowledge of how changes in circulating lymphocyte populations may relate to pulmonary infection in CF [11]. In this study we performed extensive phenotyping of peripheral blood mononuclear cell populations (PBMCs) obtained from subjects with CF and compared these profiles to those in healthy, age matched, controls. Our particular focus was on the correlation of γ/δ and MAIT innate T-cell values with clinical and microbiological parameters.

Methods

Participants and sample collection

Forty-one subjects with CF attending the Adult CF Centre, The Prince Charles Hospital, Queensland, Australia and 22 age-matched, non-smoking, healthy control subjects each supplied a single venous blood sample.

In subjects with CF, total white cell count (WCC), C-reactive protein (CRP) and clinical demographics including, age, CF genotype, lung function, body mass index, pulmonary exacerbation frequency and pulmonary pathogens (based on standard sputum microbiological testing) were recorded. Longitudinal rate of decline in forced expiratory volume in one second (FEV_1) was determined in CF subjects by means of linear regression analysis (limited to subjects with at least five FEV_1 measurements recorded over a minimum surveillance period of 2 years).

To explore the effect of pulmonary exacerbations on lymphocyte concentrations a sub-set of 13 "stable" CF subjects (stable respiratory symptoms and a CRP <5 mg L^{-1} at time of blood collection) were compared to eight CF subjects in whom blood was collected within 72 hours of admission to hospital for the intravenous antibiotic treatment of a "pulmonary exacerbation", defined as increased respiratory symptoms (cough, sputum volume or purulence, dyspnoea) (Figure S1 in File S1).

Ethics approval was obtained from The Prince Charles Hospital, Queensland, Australia, Human Research and Ethics Committee (HREC/11/QPCH/36 and HREC2008:2885) and all subjects provided written, informed consent.

Separation and storage of peripheral blood mononuclear cells (PBMCs)

Twelve millilitres of venous blood was collected into lithium heparinised tubes and separated into plasma and cellular components. The cellular component was re-suspended in RPMI (Gibco)+2% heat inactivated Foetal Calf Serum (HiFCS) and PBMCs separated by means of Histopaque 1.077 (Sigma-Aldrich) density gradient separation, as per the manufacturer's protocol.

Table 1. Subject demographics.

	Healthy Controls	Cystic Fibrosis	P value
Sex (Female:Male)	11:11	17:24	0.5
Age (years)	26 (25–32)	28 (22–32)	0.9
BMI (kg/metre^2)	24.6 (21.4–28.3)	23.5 (20.5–25.8)	0.3
FEV_1 (Litres)	4.1 (3.5–4.7)	2.2 (1.4–2.5)	<0.001
FEV_1% Predicted (%)	110 (101–120)	58 (37–75)	<0.001
FVC (Litres)	4.9 (4.4–5.7)	3.1 (2.6–4.1)	<0.001
FVC % Predicted (%)	107 (95–112)	76 (58–84)	<0.001
CFTR Genotype			
F508del Homozygotes		20	
F508del Heterozygotes		18	
Other mutations		3	
Sputum Microbiology*			
Pseudomonas aeruginosa		36	
Staphylococcus aureus		12	
Aspergillus fumigatus		5	
Haemophilus influenzae		4	
Stenotrophomonas maltophilia		3	
Chryseobacterium indologenes		2	
Scedosporium apiospermum		2	
Mycobacterium intracellulare		1	
Burkholderia gladioli		1	
Achromobacter xylosoxidans		1	
Methicillin resistant *S. aureus*		1	

Data presented as median (interquartile range).
*Summary data, subjects may have had more than one pathogen isolated in sputum, individual microbiological data available in Table S1 in File S2.
doi:10.1371/journal.pone.0109891.t001

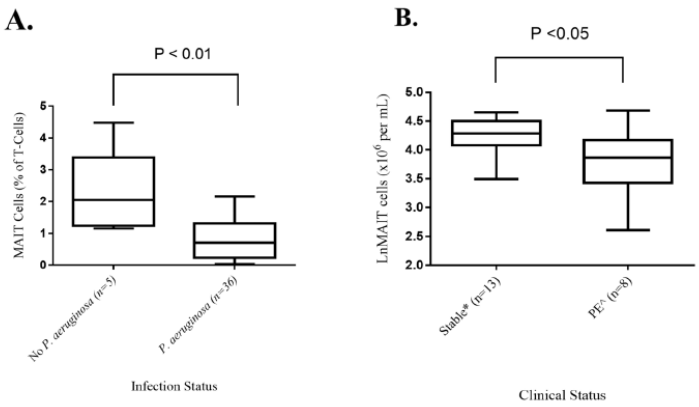


Figure 1. MAIT Cell percentage in CF subjects based on A. Presence of *P. aeruginosa* in sputum cultures, B. Clinical status. PE: pulmonary exacerbations, *two and ^one not infected with *P. aeruginosa*, between group differences determined by Mann-Whitney U test.
doi:10.1371/journal.pone.0109891.g001

Following separation, PBMCs were washed twice, re-suspended in RPMI and 15% dimethyl sulfoxide (DMSO), gradually frozen to −80°C and transferred to storage in liquid nitrogen for later batch analysis (concentration 5–20×10^6 cells/ml).

Flow Cytometry

PBMCs were rewarmed and re-suspended by drop-wise addition of 10 ml of RPMI+2%HiFCS, washed twice and re-suspended in phosphate buffered saline (PBS) with 1% HiFCS (FACS buffer). A cell count was performed and the volume adjusted to obtain a cell concentration of 10×10^6 cells/ml.

One hundred micro-litre aliquots of cells were incubated with each of two antibody staining panels as follows:

Panel 1 [adapted from [12]]: FITC anti-human CD16, Pacific Blue anti-human CD14, APC anti-human CD1c, Alexa Fluor700 anti-human CD3, APC/Cy7 anti-human HLA-DR, PE/Cy7 anti-human CD56, PE/Cy7 anti-human CD20, Anti-CD8 antibody – PE Texas (Abcam) and V500 anti-CD4 (BD biosciences) were added to 100 µL of cells and incubated in the dark for 15 minutes. Cells were washed twice in 1 ml of FACS buffer and fixation was performed by incubation with 500 µl of Cytofix (BD biosciences) for 10 minutes. Finally, samples were washed and suspended in 300 µl of FACS buffer.

Table 2. Comparison of lymphocyte sub-sets between CF and healthy control subjects.

Lymphocyte Population (size gated, CD14−)	CF (n = 41)	Non-CF (n = 22)	P-Value
T-Cells (CD3+CD16−)	**72.6 (68.1–79.6)**	**74.9 (67.9–79.0)**	0.6
CD4+CD8−	65.2 (56.1–70.5)	65.1 (59.9–71.4)	0.6
CD8+CD4−	25.7 (21.1–32.5)	27.4 (22.7–32.8)	0.6
MAIT Cells (CD161+, TCR Va7.2+)	1.1 (0.4–1.9)	2.0 (1.4–3.1)	**0.002**
γ/δ T-cells (TCR γ/δ+)	10.4 (6.5–13.4)	6.4 (4.6–9.4)	**0.012**
CD4+CD8+	0.3 (0.2–1.0)	0.4 (0.3–1.7)	0.1
CD4−CD8−*	2.1 (0.9–3.3)	1.6 (1.1–2.3)	0.3
B-Cells (CD3−CD16−CD20+HLA-DR+)	**9.5 (4.9–14.3)**	**4.8 (3.9–8.7)**	0.1
Non-Resting (CD1c−)	64.6 (57.0–74.4)	71.6 (60.5–75.1)	0.5
Resting (CD1c+)	35.0 (24.8–43.	28.1 (25.0–38.3)	0.4
NK-Cells (CD3−)	**9.5 (6.9–12.2)**	**13.1 (8.0–18.0)**	**0.013**
CD16+CD56dim HLA-DR−	75.1 (61.0–86.7)	88.0 (84.7–92.6)	**0.001**
CD16−CD56+HLA-DR−	8.9 (6.1–13.2)	6.1 (4.5–9.1)	**0.016**
CD16−CD56+HLA-DR+	14.4 (5.1–24.1)	4.3 (2.7–9.2)	**0.005**
CD3+CD16+	**2.2 (1.1–3.8)**	**1.8 (1.1–3.6)**	0.7
Contaminants/Undefined	**3.7 (3.0–5.2)**	**2.4 (1.9–3.9)**	**0.013**

*After exclusion of MAIT and γ/δ T-cells. Values expressed as percentage of parent population, Median (interquartile range). Significance of between group differences determined by Mann-Whitney U test.
doi:10.1371/journal.pone.0109891.t002

A.

	LnMAIT Cell Concentration (cells per mL)	γ/δ T-Cell Concentration (cells per mL)
FEV_1% predicted	r = 0.47 p = 0.003	r = 0.35 p = 0.030
FEV_1% predicted decline per year	r = 0.28 p = 0.10	r = 0.31 p = 0.06
FVC% predicted	r = 0.43 p = 0.006	r = 0.33 p = 0.042
Body Mass Index (kg/m^2)	r = 0.12 P = 0.48	r = -0.03 P = 0.86
C-reactive protein (mg/L)	r = -0.39 p = 0.021	r = -0.44 p = 0.008

B.

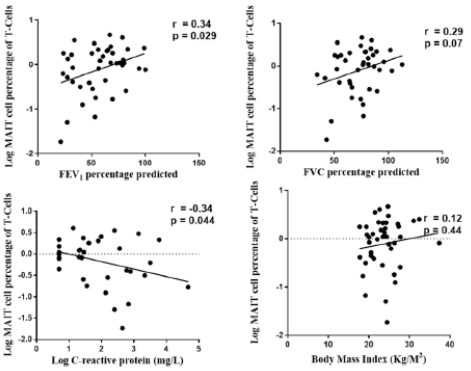


Figure 2. Relationship between MAIT and γ/δ T-cell counts and percentages with lung function. A. Pearson's Correlation co-efficient (r) and significance value (p) of MAIT and γ/δ T-cells and B. Correlation plots for MAIT Cell expressed as percentage of T-cell population, with FEV_1 and FVC % predicted, C-reactive protein and body mass index. MAIT: Mucosal invariant T-Lymphocytes, γ/δ T-Cell: Gamma-Delta T-lymphocytes, FEV_1: Forced expiratory volume in one second, FVC: Forced vital capacity.
doi:10.1371/journal.pone.0109891.g002

Table 3. Lymphocyte sub-set phenotypes in CF subjects based on the presence of *P. aeruginosa* infection compared to healthy control subjects.

	CF, No *P. aeruginosa* (n = 5)	CF, *P. aeruginosa* infection (n = 36)	P-Value^	Non-CF (n = 22)	P-Value$
Sex (Female:Male)	3:2	14:22	0.4	11:11	1.0
Age (years)	23.3 (18.4–41.7)	28 (24.5–32.6)	0.8	26 (25–32)	0.5
BMI ($kg/metre^2$)	24.7 (19.9–31.7)	23.4 (20.5–25.3)	0.5	24.6 (21.4–28.3)	0.8
FEV_1% Predicted (%)	72.4 (59.1–88.8)	63.6 (43.4–80.1)	0.3	110 (101–120)	**0.001**
FVC % Predicted (%)	83.9 (68.1–95.9)	79.3 (71.1–88.7)	0.5	107 (95–112)	**0.012**
Lymphocyte Population (size gated, CD14−)					
T-Cells (CD3+CD16−)	**68.2 (63.4–76.8)**	**72.9 (68.3–81.3)**	0.4	**74.9 (67.9–79.0)**	0.3
CD4+CD8−	64.4 (49.6–70.0)	65.2 (56.9–70.5)	0.6	65.1 (59.9–71.4)	0.5
CD8+CD4−	26.2 (22.8–38.8)	25.1 (20.5–33.1)	0.6	27.4 (22.7–32.8)	0.9
MAIT Cells (CD161+, TCR Va7.2+)	2.4 (1.4–3.6)	1.0 (0.3–1.8)	**0.023**	2.0 (1.4–3.1)	1.0
γ/δ T-cells (TCR γ/δ+)	8.0 (6.0–16.0)	10.5 (6.6–13.4)	0.7	6.4 (4.6–9.4)	0.2
CD4+CD8+	0.2 (0.2–0.2)	0.3 (0.2–1.1)	**0.014**	0.4 (0.3–1.7)	**0.004**
CD4−CD8−*	3.1 (1.6–4.1)	2.1 (0.7–2.8)	0.2	1.6 (1.1–2.3)	0.1
B-Cells (CD3−CD16−CD20+HLA-DR+)	**17.2 (14.0–20.9)**	**8.5 (3.6–13.0)**	**0.002**	**4.8 (3.9–8.7)**	**0.003**
Non-Resting (CD1c−)	74.5 (69.1–79.0)	64.1 (55.5–73.7)	0.1	71.6 (60.5–75.1)	0.1
Resting (CD1c+)	24.9 (20.3–30.5)	35.9 (25.3–43.9)	0.1	28.1 (25.0–38.3)	0.1
NK-Cells (CD3−)	**9.6 (4.6–10.9)**	**9.5 (7.8–12.5)**	**0.5**	**13.1 (8.0–18.0)**	**0.039**
CD16+CD56dim HLA-DR−	69.2 (48.5–80.9)	76.1 (63.8–88.1)	0.2	88.0 (84.7–92.6)	**0.006**
CD16−CD56+HLA-DR−	10.9 (7.1–14.5)	8.5 (6.1–13.3)	0.5	6.1 (4.5–9.1)	**0.1**
CD16−CD56+HLA-DR+	21.4 (10.8–37.4)	14.2 (4.5–23.8)	0.2	4.3 (2.7–9.2)	**0.006**
CD3+CD16+	**2.0 (0.9–2.6)**	**2.5 (1.0–4.2)**	0.4	**1.8 (1.1–3.6)**	0.9
Contaminants/Undefined	**1.8 (1.6–3.5)**	**3.7 (3.1–5.4)**	0.017	**2.4 (1.9–3.9)**	**0.4**

^CF No *P. aeruginosa versus* CF *P. aeruginosa* infection. $CF No *P. aeruginosa versus* Non-CF. *After exclusion of MAIT and γ/δ T-cells. Values expressed as percentage of parent population, Median (interquartile range). Significance of between group differences determined by Mann-Whitney U test.
doi:10.1371/journal.pone.0109891.t003

Table 4. Lymphocyte sub-sets in CF subjects based on clinical stability and compared to healthy control subjects.

	CF, Pulmonary exacerbation (n = 8)	CF, Stable (n = 13)	P-Value^	Non-CF (n = 22)	P-Value$
Sex (Female:Male)	1:7	8:5	**0.027**	11:11	0.5
Age (years)	32.2 (30.1–39.1)	22.7 (20.5–26.9)	**0.001**	26 (25–32)	**0.010**
BMI (kg/metre^2)	24.6 (23.5–26.0)	23.0 (21.5–27.0)	0.4	24.6 (21.4–28.3)	0.7
FEV_1% Predicted (%)	49.7 (40.3–68.5)	83.2 (74.1–88.8)	**0.001**	110 (101–120)	**<0.001**
FVC % Predicted (%)	74.6 (71.2–79.3)	90.0 (85.9–96.4)	**0.001**	107 (95–112)	**0.001**
Lymphocyte Population (size gated, CD14−)					
T-Cells (CD3+CD16−)	**71.6 (68.1–82.3)**	**71.3 (67.1–77.1)**	0.6	**74.9 (67.9–79.0)**	0.3
CD4+CD8−	65.9 (52.2–76.2)	58.3 (55.3–68.9)	0.5	65.1 (59.9–71.4)	0.1
CD8+CD4−	23.8 (16.4–37.8)	29.2 (23.6–35.3)	0.3	27.4 (22.7–32.8)	0.7
MAIT Cells (CD161+, TCR Va7.2+)	1.1 (0.3–2.1)	1.1 (0.9–1.9)	0.7	2.0 (1.4–3.1)	**0.018**
γ/δ T-cells (TCR γ/δ+)	11.1 (7.1–16.5)	10.5 (6.4–13.6)	0.8	6.4 (4.6–9.4)	**0.017**
CD4+CD8+	0.2 (0.2–1.0)	0.2 (0.2–0.3)	0.5	0.4 (0.3–1.7)	**0.010**
CD4−CD8−*	2.6 (1.4–3.5)	2.2 (1.3–3.4)	0.6	1.6 (1.1–2.3)	0.1
B-Cells (CD3−CD16−CD20+HLA-DR+)	**9.6 (2.5–14.0)**	**13.1 (8.5–16.7)**	0.3	**4.8 (3.9–8.7)**	**0.001**
Non-Resting (CD1c−)	73.4. (65.5–79.9)	70.4 (59.9–79.9)	0.8	71.6 (60.5–75.1)	0.7
Resting (CD1c+)	25.6 (19.9–34.1)	29.3 (18.1–40.1)	0.8	28.1 (25.0–38.3)	0.7
NK-Cells (CD3−)	**10.1 (5.9–12.6)**	**9.6 (5.4–11.8)**	0.7	**13.1 (8.0–18.0)**	**0.020**
CD16+CD56dim HLA-DR−	70.7 (52.9–83.7)	69.2 (55.3–80.5)	0.9	88.0 (84.7–92.6)	**<0.001**
CD16−CD56+HLA-DR−	9.9 (8.6–12.6)	8.0 (6.7–13.3)	0.6	6.1 (4.5–9.1)	**0.026**
CD16−CD56+HLA-DR+	19.4 (5.8–37.2)	21.4 (13.2–30.6)	0.8	4.3 (2.7–9.2)	**<0.001**
CD3+CD16+	**2.0 (1.2–4.0)**	**2.5 (1.1–4.2)**	1.0	**1.8 (1.1–3.6)**	0.7
Contaminants/Undefined	**3.6 (3.1–4.2)**	**3.5 (2.5–4.1)**	0.6	**2.4 (1.9–3.9)**	**0.4**

^CF pulmonary exacerbation *versus* CF stable, $CF stable *versus* Non-CF. *After exclusion of MAIT and γ/δ T-cells. Values expressed as percentage of parent population, Median (interquartile range). Significance of between group differences determined by Mann-Whitney U test.

doi:10.1371/journal.pone.0109891.t004

Panel 2: Surface staining was performed by incubation with V500 anti-CD4, FITC anti-TCR Va7.2, PerCP/Cy5.5 anti-CD161, APC/Cy7 anti-CD3, PE anti-TCR γ/δ, PE Texas red Anti-CD8 for 15 minutes at room temperature in the dark. Cells were washed twice in 1 ml of FACS buffer, fixed and resuspended in 300 µl of buffer solution.

Unless stated otherwise, antibodies were obtained for Biolegend, San Diego. Antibody titration was performed to optimise antibody-cell concentration prior to testing.

Sample analysis was performed on a Fortessa IV flow cytometer (BD Biosciences). A lymphocyte gate was set based on forward and side scatter properties and a minimum of 50,000 gated events were capture for each sample.

MAIT cells were defined as $CD3^+/CD4^-/CD8^+$or−/$CD161^+$/TCR $Va7.2^+$ [13].

For subjects with CF, automated haemocytometer, absolute lymphocyte counts were obtained and T-cell sub-types were considered as both, absolute numbers of cells per mL of blood and percentage of the whole T-cell population.

Data analysis was performed using Flowjo version 7.6 (Treestar), representative gating plots are available in Figure S2 in File S1.

Statistical Analysis

Statistical analysis was performed using PASW, version 18 (SPSS Inc. Chicago IL, USA) and Graph-pad Prism, version 6. Between group differences in PBMC populations were examined using student's t-test or Mann-Whitney U test. Shapiro-Wilk test and q-q plots were used to determine normal distribution of continuous variables. Non-normally distributed continuous variables (WCC, CRP, MAIT cell absolute count and percentage, B-cell count, NKT-cell and NK-cell percentage) were natural logarithm transformed and Pearson's correlation used to determine relationships between variables. A p-value<0.05 was considered to represent statistical significance.

Results

The characteristics of subjects with CF and healthy controls are provided in Table 1. Thirty-six of the 41 subjects with CF had chronic pulmonary infection with *P. aeruginosa* (either in isolation or in combination with another CF respiratory pathogen) on routine microbiological cultures. The remaining five subjects did not have *P. aeruginosa* infection on current, or previous sputum cultures (Table S1 in File S1 for complete sputum microbiological data).

A greater number of subjects in the pulmonary exacerbation group were male, these subjects were also older, with more severe lung disease, when compared to the stable subjects.

Comparison of lymphocyte sub-sets between groups, demonstrated a reduction in the percentage of MAIT cells in subjects with CF, compared to healthy controls (median 1.1% versus 2.0%, $p = 0.002$), with an accompanying increase in the percentage of γ/δ T-cells (median 10.4% versus 6.4%, $p = 0.012$). CF subjects also displayed reduced percentages of NK-cells (median 9.5% versus

13.1%, p = 0.013). The percentage of cells in all of the other major lymphocyte sub-sets was similar between groups (Table 2).

Relationship between MAIT cells, microbiological and clinical parameters in subjects with CF

Absolute MAIT cell concentrations and the proportion of T-cells that were MAIT cells (MAIT cell percentage), in the five subjects without *P. aeruginosa* infection were significantly higher than in patients with chronic *P. aeruginosa* infection (Table 3 and Figure 1A). MAIT cell percentages in subjects not infected with *P. aeruginosa* were similar to healthy controls subjects. No difference was seen in the MAIT cell percentage of subjects with a *P. aeruginosa* infection, based on their co-pathogen (Figure S3 in File S1).

Absolute blood MAIT cell counts in the sub-group of stable CF subjects were higher, when compared to subjects sampled early in the course of treatment for a pulmonary exacerbation (Figure 1B). However, the MAIT cell percentage was similar between stable and pulmonary exacerbation subjects (Table 4).

Absolute MAIT cell count and MAIT cell percentage correlated positively with FEV_1 and FVC percentage predicted. A weak relationship was seen between increased rate of FEV_1 decline and MAIT cells counts, but this did not reach statistical significance (Figure 2).

MAIT cell percentage and absolute MAIT cell concentrations were inversely correlated with CRP ($r = -0.34$, $p = 0.044$ and $r = -0.39$, $p = 0.021$, respectively), however, there was no relationship between MAIT cells and total WCC.

Relationship between γ/δ T-cells, microbiological and clinical parameters in subjects with CF

Absolute γ/δ T-cell counts correlated with FEV_1 and FVC percentage predicted values. A trend toward more rapid rate of lung function decline in FEV_1 was seen in subjects with lower γ/δ T-cells numbers (Figure 2A). No relationship between γ/δ T-cells when expressed as a percentage of total T-cells and lung function parameters was observed (Figure 2B).

γ/δ T-cell percentage and absolute cell number were inversely correlated with CRP ($r = -0.44$, $p = 0.008$) and absolute γ/δ T-cell number was higher in stable CF subjects compared to subjects experiencing a pulmonary exacerbation (Figure S4 in File S1).

There was no relationship between γ/δ T-cell counts or percentages and absolute WCC or profile of infection with *P. aeruginosa* (Table 3).

Relationship between other lymphocyte sub-subset and clinical parameter

Absolute blood lymphocyte count was positively correlated with FEV_1 and FVC (litres and percentage predicted), however, no relationship was seen between absolute WCC and the percentage of the major lymphocyte sub-sets (T-cells, B-cells, NK-cells, CD3+/CD16+ cells) or lung function (Table S2 in File S1).

Total lymphocyte count was decreased in CF subjects being treated for a pulmonary exacerbation.

B-cell percentage was higher in subjects not infected with *P. aeruginosa*, compared to CF subjects with chronic *P. aeruginosa* infection and healthy controls. There was a trend towards lower B-cell percentages in CF subjects during a pulmonary exacerbation compared to stable CF patients (Figure S5A and B in File S1).

Discussion

In this study we demonstrate for the first time that the peripheral blood of subjects with CF is characterized by a relative lymphopenia and major reductions in circulating MAIT cells and to a lesser extent an increase in γ/δ T-cells compared to normal healthy controls, consistent with both quantitative and qualitative differences in innate T-cell immunity in CF. Importantly, lung disease severity, systemic inflammation, clinical status and the presence or absence of chronic *P. aeruginosa* infection were all significantly related to the number of circulating MAIT cells in peripheral blood.

The reduction in absolute lymphocyte counts as lung disease severity increases in the current study is consistent with a single previous study of peripheral blood lymphocytes in children with CF [11]. Similar to this earlier study, a reduction in the percentage of NK-cells was also seen in subjects with CF, however, in contrast, no difference in the percentage of CD4+ T cells was seen and the percentages of other major lymphocyte subsets were similar between CF subjects and healthy control subjects [11]. NK-cells represent an innate, cytotoxic subset of lymphocyte which primarily respond to viral infections and tumour cells [14]. In addition, NK cells provide critical support to the eradication of bacterial pulmonary infection (including *P. aeruginosa*), principally through the generation of Th2 cytokines and IFN-γ [15,16].

A higher percentage of B-cells was seen in CF subjects not infected with *P. aeruginosa* and a trend towards lower concentrations in subjects who were undergoing treatment for an acute pulmonary exacerbation. B-cells are critical for pulmonary protection against encapsulated bacteria and B-cells cultured *in vitro* respond to the mucoid exopolysaccharide of *P. aeruginosa* [17]. Further longitudinal studies are required to delineate the role of B-cells during acute pulmonary exacerbations and to determine whether changes in circulating numbers can be attributed to peripheral destruction, tissue sequestration or transformation to plasma cells.

The association between MAIT cells counts and *P. aeruginosa* infection, severe lung function impairment, increased systemic inflammation and acute pulmonary exacerbations in subjects with CF may suggest that MAIT cell deficiency is associated with susceptibility to pulmonary infection in CF. However, the sub-group of CF subjects undergoing a pulmonary exacerbation may have been biased towards a lower MAIT cell concentration, as more subjects in this sub-group were male and in general they had poorer baseline lung function [18]. Furthermore, these data are cross-sectional and do not inform on whether low MAIT cell numbers contribute to *P. aeruginosa* colonisation and disease progression, or whether low numbers simply reflect depletion of MAIT cells in the circulation, because of recruitment to the airway mucosa.

MAIT cells provide a pivotal link between the innate and adaptive immune responses. The semi-invariant T-cell receptor on MAIT cells recognises metabolite derivatives of pathogens (e.g. vitamin B (riboflavin and folic acid)) presented combined to MR-1 [19]. Activated MAIT cells produce high concentrations of pro-inflammatory interleukin (IL)-17 [20], which invokes a potent cascade of cytokines and chemokines (e.g. IL-8 and G-CSF) that promote neutrophil migration into the airways. Interleukin-17 is involved in neutrophil recruitment in CF and high concentrations have been described in sputum and bronchoalveolar lavage fluid of subjects with CF infected with *P. aeruginosa* and also in those patients with severe disease [21–23].

Mouse models demonstrate MAIT cell recruitment into the lungs at a very early stage of bacterial infection and sustained

MAIT cell responsiveness during the late stages of infection contributes towards an ongoing cytokine response [10]. In mice with selective MAIT cell deficiency the immune response was ineffective in controlling pulmonary infection [10].

Whilst we provide the first description of circulating MAIT cells in CF, understanding of the role of MAIT cells in human disease is limited. A reduction in the proportion of circulating MAIT cells has been reported in subjects with human immunodeficiency virus (HIV), *Mycobacterium tuberculosis* (MTB) and other severe pulmonary infections, and sepsis [7,8,24–26]. In HIV infection, blood MAIT cells concentrations decrease progressively from time of infection, however, MAIT cell density remains relatively preserved in rectal mucosa, suggesting possible preferential recruitment and thus loss from the circulating pool [27,28]. Similarly, MTB reactive MAIT cells are enriched in lung lymph of healthy people when compared to matched blood samples [29]. Collectively, the results of these earlier studies and our current work suggest that although blood MAIT cell counts may not reflect tissue concentrations, they may still prove to be a useful, surrogate biomarker of the immune response within the lung [30]. We demonstrate a strong, inverse relationship between MAIT cell counts and CRP and the specificity of MAIT cell responsiveness to bacterial and fungal infections, offers the potential for improved specificity compared to CRP, which will respond to both viruses and non-infective sources of systemic inflammation [31]. Further mechanistic studies of MAIT cells in CF, including assessment in the actual airway will provide novel insights into their role in innate and adaptive immunity in the CF lung, including whether effective vaccines can be developed that boost MAIT cell function to allow eradication of key bacterial pathogens.

γ/δ T-cells represent a minor population of circulating T-cells, which has the capacity to expand rapidly in response to bacterial infection [32]. γ/δ T-and MAIT cells share many similarities; they both produce IL-17 and preferentially migrate to mucosal surfaces from the circulation [33]. In murine pulmonary infection models, γ/δ T-cells rapidly accumulate in the lung, in response to a range bacterial pathogens, where they facilitate the influx of neutrophils and subsequent bacterial clearance [32]. The small increase in the proportion of γ/δ T-cells in the blood of CF subjects in the current study is consistent with findings of a single previous study in CF [34]. However, the relationship between γ/δ T-cells and clinical parameters in CF subjects were only significant when absolute γ/δ T-cell concentrations were considered and these relationships may simply reflect changes in the absolute numbers of T-cells, rather than any implying any specific role for γ/δ T-cell in pathology.

Our current study has several limitations. Firstly, cross-sectional data collection, does not inform whether a causal relationships exists between lymphocytes subsets and clinical parameters. Furthermore, the limited number of patients included in the analysis of stable disease *versus* pulmonary exacerbation, and inclusion of only five patients not infected with *P. aeruginosa* means type I statistical errors are possible. Longitudinal studies which collect blood samples from the same subject before and after the acquisition of *P. aeruginosa,* or during a pulmonary exacerbation and again during a period of stable disease are required. Finally, blood lymphocyte population may not reflect airway populations and studies which correlate lymphocyte concentrations in airway biopsies to those in the blood are anticipated.

Conclusion

In summary, we describe important differences in the proportions of circulating MAIT and γ/δ T-cell in adult patients with CF, compared to healthy control subjects. Reduced numbers of MAIT cells were associated with *P. aeruginosa* pulmonary infection and more severe lung disease. Our findings provide the impetus for future studies examining the utility of MAIT cells in immunotherapies and vaccine development, and longitudinal studies of MAIT cells as biomarkers of CF pulmonary infection.

Supporting Information

File S1 Tables S1, S2, and Figures S1–S5. Table S1 in File S1. Sputum microbiology of CF subjects. Table S2 in File S1. Relationship between White cell count and lymphocyte subsets, and C-reactive protein, body mass index and lung function. Figure S1 in File S1 Flow diagram of subjects include in sub-group analysis. Figure S2 in File S1 Representative flow-cytometry gating plots. Figure S3 in File S1. MAIT cell percentage in CF subjects with *Pseudomonas aeruginosa* and a co-pathogen in sputum culture. Figure S4 in File S1. Comparison of γ/δ T-cell counts in stable subjects and subjects undergoing antibiotic treatment for a pulmonary exacerbation. Figure S5 in File S1. Comparison of lymphocyte counts and percentage of lymphocyte sub-sets between A. Stable and pulmonary exacerbations B. *P. aeruginosa* infected and non-infected CF subjects.
(DOC)

File S2 Individual Subject Raw Data.
(XLS)

Author Contributions

Conceived and designed the experiments: DS DR GH SB. Performed the experiments: DS. Analyzed the data: DS. Contributed reagents/materials/analysis tools: DS DR GH. Contributed to the writing of the manuscript: DS DR GH SB.

References

1. Elizur A, Cannon CL, Ferkol TW (2008) Airway inflammation in cystic fibrosis. Chest 133: 489–495.
2. Ratner D, Mueller C (2012) Immune responses in Cystic Fibrosis; are they intrinsically defective? Am J Respir Cell Mol Biol.
3. Regamey N, Tsartsali L, Hilliard TN, Fuchs O, Tan HL, et al. (2012) Distinct patterns of inflammation in the airway lumen and bronchial mucosa of children with cystic fibrosis. Thorax 67: 164–170.
4. Moser C, Jensen PO (2011) Adaptive Immune Responses and Biofilm Infections. Biofilm Infections: 201–214.
5. Hartl D, Griese M, Kappler M, Zissel G, Reinhardt D, et al. (2006) Pulmonary T(H)2 response in Pseudomonas aeruginosa-infected patients with cystic fibrosis. J Allergy Clin Immunol 117: 204–211.
6. Moser C, Kjaergaard S, Pressler T, Kharazmi A, Koch C, et al. (2000) The immune response to chronic Pseudomonas aeruginosa lung infection in cystic fibrosis patients is predominantly of the Th2 type. APMIS 108: 329–335.
7. Gold MC, Lewinsohn DM (2013) Co-dependents: MR1-restricted MAIT cells and their antimicrobial function. Nat Rev Microbiol 11: 14–19.
8. Le Bourhis L, Martin E, Peguillet I, Guihot A, Froux N, et al. (2010) Antimicrobial activity of mucosal-associated invariant T cells. Nat Immunol 11: 701–708.
9. Lanier LL (2013) Shades of grey–the blurring view of innate and adaptive immunity. Nat Rev Immunol 13: 73–74.
10. Meierovics A, Yankelevich WJ, Cowley SC (2013) MAIT cells are critical for optimal mucosal immune responses during in vivo pulmonary bacterial infection. Proc Natl Acad Sci U S A 110: E3119–3128.
11. Hausler M, Schweizer K, Biesterfel S, Opladen T, Heimann G (2002) Peripheral decrease and pulmonary homing of CD4+CD45RO+ helper memory T cells in cystic fibrosis. Respir Med 96: 87–94.
12. Autissier P, Soulas C, Burdo TH, Williams KC (2010) Evaluation of a 12-color flow cytometry panel to study lymphocyte, monocyte, and dendritic cell subsets in humans. Cytometry A 77: 410–419.
13. Martin E, Treiner E, Duban L, Guerri L, Laude H, et al. (2009) Stepwise development of MAIT cells in mouse and human. PLoS Biol 7: e54.
14. Hamerman JA, Ogasawara K, Lanier LL (2005) NK cells in innate immunity. Curr Opin Immunol 17: 29–35.

15. Culley FJ (2009) Natural killer cells in infection and inflammation of the lung. Immunology 128: 151–163.
16. Wesselkamper SC, Eppert BL, Motz GT, Lau GW, Hassett DJ, et al. (2008) NKG2D is critical for NK cell activation in host defense against Pseudomonas aeruginosa respiratory infection. J Immunol 181: 5481–5489.
17. Daley L, Pier GB, Liporace JD, Eardley DD (1985) Polyclonal B cell stimulation and interleukin 1 induction by the mucoid exopolysaccharide of Pseudomonas aeruginosa associated with cystic fibrosis. J Immunol 134: 3089–3093.
18. Novak J, Dobrovolny J, Novakova L, Kozak T (2014) The decrease in number and change in phenotype of mucosal-associated invariant T cells in the elderly and differences in males and females of reproductive age. Scand J Immunol.
19. Lopez-Sagaseta J, Dulberger CL, McFedries A, Cushman M, Saghatelian A, et al. (2013) MAIT recognition of a stimulatory bacterial antigen bound to MR1. J Immunol 191: 5268–5277.
20. Dusseaux M, Martin E, Serriari N, Peguillet I, Premel V, et al. (2011) Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161hi IL-17-secreting T cells. Blood 117: 1250–1259.
21. McAllister F, Henry A, Kreindler JL, Dubin PJ, Ulrich L, et al. (2005) Role of IL-17A, IL-17F, and the IL-17 receptor in regulating growth-related oncogene-alpha and granulocyte colony-stimulating factor in bronchial epithelium: implications for airway inflammation in cystic fibrosis. J Immunol 175: 404–412.
22. Tan HL, Regamey N, Brown S, Bush A, Lloyd CM, et al. (2011) The Th17 pathway in cystic fibrosis lung disease. Am J Respir Crit Care Med 184: 252–258.
23. Tiringer K, Treis A, Fucik P, Gona M, Gruber S, et al. (2013) A Th17- and Th2-skewed cytokine profile in cystic fibrosis lungs represents a potential risk factor for Pseudomonas aeruginosa infection. Am J Respir Crit Care Med 187: 621–629.
24. Grimaldi D, Le Bourhis L, Sauneuf B, Dechartres A, Rousseau C, et al. (2014) Specific MAIT cell behaviour among innate-like T lymphocytes in critically ill patients with severe infections. Intensive Care Med 40: 192–201.
25. Wong EB, Akilimali NA, Govender P, Sullivan ZA, Cosgrove C, et al. (2013) Low levels of peripheral CD161++CD8+ mucosal associated invariant T (MAIT) cells are found in HIV and HIV/TB co-infection. PLoS One 8: e83474.
26. Jiang J, Wang X, An H, Yang B, Cao Z, et al. (2014) Mucosal-associated Invariant T-Cell Function Is Modulated by Programmed Death-1 Signaling in Patients with Active Tuberculosis. Am J Respir Crit Care Med 190: 329–339.
27. Cosgrove C, Ussher JE, Rauch A, Gartner K, Kurioka A, et al. (2013) Early and nonreversible decrease of CD161++/MAIT cells in HIV infection. Blood 121: 951–961.
28. Leeansyah E, Ganesh A, Quigley MF, Sonnerborg A, Andersson J, et al. (2013) Activation, exhaustion, and persistent decline of the antimicrobial MR1-restricted MAIT-cell population in chronic HIV-1 infection. Blood 121: 1124–1135.
29. Gold MC, Cerri S, Smyk-Pearson S, Cansler ME, Vogt TM, et al. (2010) Human mucosal associated invariant T cells detect bacterially infected cells. PLoS Biol 8: e1000407.
30. Shoki AH, Mayer-Hamblett N, Wilcox PG, Sin DD, Quon BS (2013) Systematic review of blood biomarkers in cystic fibrosis pulmonary exacerbations. Chest 144: 1659–1670.
31. Levy H, Kalish LA, Huntington I, Weller N, Gerard C, et al. (2007) Inflammatory markers of lung disease in adult patients with cystic fibrosis. Pediatr Pulmonol 42: 256–262.
32. Ito M, Kojiro N, Ikeda T, Ito T, Funada J, et al. (1992) Increased proportions of peripheral blood gamma delta T cells in patients with pulmonary tuberculosis. Chest 102: 195–197.
33. Sutton CE, Mielke LA, Mills KH (2012) IL-17-producing gammadelta T cells and innate lymphoid cells. Eur J Immunol 42: 2221–2231.
34. Raga S, Julia MR, Crespi C, Figuerola J, Martinez N, et al. (2003) Gammadelta T lymphocytes from cystic fibrosis patients and healthy donors are high TNF-alpha and IFN-gamma-producers in response to Pseudomonas aeruginosa. Respir Res 4: 9.

Journal of Cystic Fibrosis 13 (2014) 289–295

www.elsevier.com/locate/jcf

Original Article

Elevated metal concentrations in the CF airway correlate with cellular injury and disease severity

Daniel J. Smith [a,b,c,*], Gregory J. Anderson [b,c], Scott C. Bell [a,b,d], David W. Reid [a,b,c]

[a] Department of Thoracic Medicine, The Prince Charles Hospital, Rode Road, Chermside, Brisbane, Queensland 4032, Australia
[b] School of Medicine, University of Queensland, 288 Herston Road, Herston, Brisbane, Queensland 4029, Australia
[c] The Iron Metabolism Laboratory, Queensland Institute of Medical Research, 300 Herston Road, Herston, Queensland 4029, Australia
[d] Queensland Children's Medical Research Institute, Royal Children's Hospital, Herston Road, Herston, Queensland 4029, Australia

Received 9 September 2013; received in revised form 1 December 2013; accepted 1 December 2013
Available online 24 December 2013

Abstract

Background: Bio-active trace metals have been identified in respiratory tract secretions of subjects with lung disease and may potentially influence bacterial virulence, inflammation and disease severity. We measured a diverse range of metal ions in sputum samples from subjects with CF and non-CF bronchiectasis (NCFB) compared to healthy controls and examined their relationship to airway inflammation, disease severity and the presence of bacterial pathogens.
Methods: We studied 45 subjects with CF, 8 with NCFB and 8 healthy controls. Metal concentrations were measured in sputum supernatant by inductively-coupled plasma mass spectrometry and correlated with sputum inflammatory cell counts, lactate dehydrogenase (LDH) and interleukin (IL)-8 concentrations, atmospheric particulate matter, lung function, clinical status and participant demographics.
Results: Sputum from subjects with CF and NCFB contained increased concentrations of magnesium, calcium, iron and zinc. Metal ion concentrations correlated positively with LDH levels. The concentrations of magnesium, iron and zinc positively correlated with IL-8. A sub-group of CF subjects with severe lung disease demonstrated increased sputum molybdenum concentrations.
Conclusion: Elevated concentrations of sputum metal ions appear to be associated with cell/tissue necrosis and inflammation in subjects with CF and NCFB. Sputum molybdenum concentrations may be a biomarker of severe CF airway disease.

Keywords: Cystic Fibrosis; Bio-metals; Inflammation; Cellular injury

1. Introduction

The majority of individuals with cystic fibrosis (CF) will die prematurely from complications associated with chronic pulmonary sepsis [1]. Intermittent airway infection and inflammation in CF begins in infancy, resulting in early onset bronchiectasis in many children [2]. By adulthood, chronic, poly-microbial infection is established, in which *Pseudomonas aeruginosa* is the dominant pathogen in more than eighty percent of patients [3]. The host immune response to infection in CF is intense and persistent, but is ineffective in clearing bacterial infection from the airways and contributes to local tissue destruction through the generation of proteolytic enzymes and reactive oxygen species (ROS) [4].

As pulmonary disease progresses, plugging of the distal airways by dehydrated sputum creates micro-aerobic or frankly anaerobic pockets, with low pH and altered nutrient availability [5,6]. The highly abnormal environment within these regions drives phenotypic adaptation and alters the virulence of the incumbent bacteria, favouring pathogens capable of survival in low oxygen environments [6].

Bio-active trace metals (biometals) are essential co-factors in a wide range of human and bacterial enzyme systems; however, strict regulation of their bioavailability is essential to prevent toxicity [7]. A limited number of previous studies in patients with CF, non-CF bronchiectasis (NCFB) and chronic bronchitis have

* Corresponding author at: Department of Thoracic Medicine, The Prince Charles Hospital, Rode Road, Chermside, Brisbane, Queensland 4032, Australia. Tel.: +61 7 3139 6770; fax: +61 7 3139 5630.
E-mail address: Daniel_j_smith@health.qld.gov.au (D.J. Smith).

1569-1993/$ -see front matter
http://dx.doi.org/10.1016/j.jcf.2013.12.001

demonstrated increased iron (Fe), zinc (Zn) and copper (Cu) concentrations in airway secretions and have postulated that these metals may influence disease severity [8,9].

There is increasing interest in Fe in the CF lung. *In vitro* studies demonstrate that Fe strongly influences the ability of *P. aeruginosa* to form co-dependent bacterial communities (biofilms), which represent the major barrier to the eradication of established airway infection. Manipulation of Fe availability has therefore been proposed as a novel therapeutic strategy for the treatment of chronic *P. aeruginosa* infection [10,11]. An observational study by Gray and colleagues has previously demonstrated increased concentrations of Zn and Cu in the CF lung [8], and in separate *in vitro* studies these metals have been shown to induce *P. aeruginosa* resistance to carbapenem antibiotics [12]. From the host perspective, airway Fe and other redox active biometals may catalyse the production of ROS and promote inflammation [13]. Conversely, Cu and Zn are key components of airway anti-inflammatory superoxide dismutases (SODs) [14]. The origin of airway metal ions has not been determined, with potential sources including vascular leak, channelopathies and release from necrotic airway cells, or the bacteria themselves.

In this study, we expand on previous work by examining a more diverse range of biometals in expectorated sputum samples from patients with CF and NCFB. Sputum biometal concentrations are compared to those in healthy controls, and correlated with sputum lactate dehydrogenase (LDH) as an index of local tissue necrosis and interleukin (IL)-8 as a marker of airway inflammation and host immune response. To examine the influence of infection and clinical status on biometal concentration, we included a sub-group of patients with CF who were not infected with *P. aeruginosa* and followed a group of *P. aeruginosa* infected patients through treatment of a pulmonary exacerbation with a course of intravenous antibiotic therapy.

Calcium (Ca), magnesium (Mg), manganese (Mn), Zn and Cu were selected for their importance in inflammatory pathways [13–15]. Molybdenum (Mo) and Fe were selected for their potential importance to bacterial virulence and anaerobic respiration [9,16]. Finally, nickel (Ni) and lead (Pb) were included as potential indicators of environmental contamination [17,18].

2. Methods

2.1. Participants and sample collection

Participants were recruited from The Prince Charles Hospital, Queensland and Royal Hobart Hospital, Tasmania, Australia. Institutional human research and ethics committee approval was gained from both sites (HREC2008:2885 and H0009813 respectively). Sixty-seven spontaneously expectorated sputum samples were collected from 45 subjects with CF and a single sputum sample was collected from eight subjects with NCFB. Induced sputum was collected from eight healthy control subjects following inhalation of nebulised 4.5% hypertonic saline. For cross-sectional analyses, the initial sputum sample collected from each subject was used. Eleven subjects with CF provided samples at several time-points during intravenous antibiotic treatment of a pulmonary exacerbation. Participant demographics, including infecting pathogens were recorded (Table 1). None of the participants reported regular tobacco smoking.

2.2. Sputum processing

Sputum plugs free of salivary contamination were separated from the expectorated sputum samples and homogenised with dithiothreitol as previously described [19]. Homogenised samples were diluted with phosphate buffered saline (dilution factor 1:10) and centrifuged to pellet cells. The cell free supernatant was removed and stored at −80 °C for later batch analysis of biometals. The cell pellet was resuspended and total inflammatory cell count (TCC) performed using standard methodology [19].

2.3. Inductively coupled plasma mass-spectrometry (ICP-MS) analysis

To prepare samples for ICP-MS, 500 µL of sputum supernatant was digested overnight in an acid-cleaned tube containing 500 µL of 3 M double-distilled nitric acid (HNO_3) and 1 mL of an internal standard spike solution (^{6}He, ^{61}Ni, ^{103}Rh, ^{115}In, ^{187}Re and ^{209}Bi). Digested samples were diluted to 10 mL with 0.3 M

Table 1
Participant demographics.

	Normal (n = 8)	CF (n = 45)	Non-CF bronchiectasis (n = 8)
Age (years)	57 (7)	29 (12)	60 (10)[b]
Sex (M:F)	6:2	28:17	4:4
FEV_1 % predicted	106 (10)	53 (23)[a]	33 (15)[c]
FVC % predicted	110 (16)	65 (21)[a]	61 (6)[c]
BMI	n/a	21 (3)[b]	25 (4)[d]
Airway pathogens (n, %)			
–*Pseudomonas aeruginosa*	–	33 (73%)	6 (75%)
–MSSA	–	16 (36%)	–
–*Aspergillus* spp.	–	9 (20%)	2 (25%)
–MRSA	–	2 (4%)	–
–*Burkholderia* spp.	–	3 (6%)	–
–*Haemophilus influenzae*	–	1 (2%)	1 (13%)

Values reported as mean (standard deviation) unless otherwise stated, M:F: Male:Female ratio, data unavailable for [a]one, [b]two, [c]three, and [d]four subjects, MSSA: methicillin sensitive *Staphylococcus aureus*, MRSA: methicillin resistant *Staphylococcus aureus*, spp.: species. BMI: Body mass index. n/a: not available.

HNO_3 and analysed using a Thermo Fischer X Series ICP-MS machine. Four replicates per sample were averaged for the final calculation. For calibration and to establish recovery rates, standard reference materials W2 and SLRS5 and multi-element standards were analysed at the beginning and the end of the run. Internal standards were used to correct for internal drift. A monitor solution, used to correct for external drift, and an instrument blank solution, used to monitor baseline drift and memory effects, were analysed at regular intervals throughout the run.

2.4. LDH and IL-8 measurement

LDH activity was determined in thawed sputum supernatants warmed to 37 °C using a LDH colorimetric assay kit (Abcam Inc., Cambridge, MA) according to the manufacturer's protocol. IL-8 (BioLegend, USA) concentration was measured according to the manufacturer's protocol with a detection range between 31.25 and 4000 pg/mL. Each sample was diluted as necessary to fall in the linear range and assayed in duplicate.

2.5. Air quality assessment

To examine whether atmospheric pollutants potentially contributed to sputum metal concentrations, we recorded the level of atmospheric particulate matter 10 μm or smaller (PM_{10}) as reported by the Queensland Department of Environment and Heritage Protection (http://www.ehp.qld.gov.au/air/data/search.php) for the city of Brisbane on the day a sputum sample was collected. These data were available for subjects sampled through the Prince Charles Hospital, but no similar data were available for Tasmanian participants.

2.6. Statistical analysis

Between-group differences in continuous variables were analysed by independent sample t-test, analysis of covariance, Mann–Whitney U or Kruskal–Wallis H test, depending on number of independent groups and normality of the data. Pearson's correlation was used to examine relationships between lung function, TCC, IL-8, LDH, PM_{10} and biometal concentrations. A p-value < 0.05 was considered to represent statistical significance. Data analysis was performed using PASW, Version 18.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Cross-sectional comparisons

Sixty-one samples (8 normal, 8 NCFB, 45 CF) were included in the cross-sectional analysis. Sputum from subjects with CF and NCFB demonstrated a high TCC and contained significantly higher concentrations of Mg, Ca, Fe and Zn compared to healthy controls (Table 1).

Sputum metal concentrations were compared to published data on biometal concentration in normal serum, whole blood and pleural fluid (Table 2) [20–24]. Sputum from subjects with CF and NCFB contained higher metal concentrations than serum and pleural fluid (except for Cu). Sputum from healthy controls contained lower concentrations of biometals than serum, pleural fluid and whole blood (except for Pb which was higher than in serum and pleural fluid).

In patients with CF, two distinct cohorts of subjects were detected with and without significant concentrations of Mo (Fig. 1). Subjects with elevated Mo concentrations had lower FEV_1 % predicted, but also lower TCC (Supplemental Table 1).

Six subjects with NCFB and 33 with CF were infected with *P. aeruginosa*. CF subjects infected with *P. aeruginosa* had lower lung function (Median FEV_1 % predicted 38% v 71%, $p < 0.01$) and higher sputum concentration of IL-8 (165 v 70 ng/mL, $p < 0.01$), Ca (105 v 74 mg/L, $p < 0.01$) and Mg (22 v 47 mg/L, $p < 0.05$) compared to subjects without *P. aeruginosa* infection; however TCCs and levels of other measured metals were not significantly different (Supplemental Table 2).

3.2. Relationship between biometals, LDH, IL-8 and PM_{10} within samples

A correlation analysis, including all sputum samples demonstrated that the concentrations of Mg, Ca, Fe, Zn and Cu were all strongly correlated with one another (Table 3). Conversely, no relationship was seen between concentrations of Pb, Mo or Ni. In sputum samples from subjects with CF and NCFB, both LDH and IL-8 concentrations positively correlated with the concentration of Mg, Fe and Zn, and LDH alone correlated with Ca and Cu concentrations (Table 3).

Comparative PM_{10} data was available for 43 sputum samples from 23 CF subjects. There was no correlation between PM_{10} and metal concentrations in sputa on the day of collection (Table 3).

3.3. Sputum biometals during pulmonary exacerbations

Four CF subjects had increased sputum Pb levels on admission, which fell to trace levels during the course of the admission. Sputum Fe concentration increased during treatment in three subjects, of which, two received intercurrent iron infusions to correct severe iron deficiency. There was no consistent change in sputum concentrations of the other metals during treatment of a pulmonary exacerbation (Supplemental Fig. 1).

4. Discussion

This work provides important new insights into the inflammatory milieu present in the lungs of patients with CF and NCFB. A diverse range of biometals were present in the sputum of subjects with these suppurative lung diseases, several of which have the potential to significantly influence both host immunity and bacterial virulence [25].

Defining the importance of the biometals we detected in sputum and how these metals relate to lung disease severity will advance our understanding of the lung micro-environment in diseases characterised by chronic bacterial infection.

Table 2
Comparison of sputum inflammatory markers and biometals in healthy controls, CF and non-CF bronchiectasis.

	Normal (n = 8)	CF (n = 45)	NCFB (n = 8)	p-Value[a]		Pleural fluid [21]	Serum [Reference]	Blood [Reference]
				CF	NCFB			
TCC (10^6 cell/mL)	0 (0–1)	12 (4–21)	24 (12–35)	<0.001	<0.01			
LDH (mU/mL)	39 (0–95)[c]	50 (1–247)[f]	13 (3–1657)[b]	0.5	0.5			
IL-8 (ng/mL)	28 (15–64)[c]	135 (84–279)[d]	103 (13–292)[b]	<0.01	0.2			
Mg (mg/L)	4 (2–7)	30 (19–44)	33 (27–39)	<0.001	<0.01	18	22 [21]	
Ca (mg/L)	45 (28–58)	102 (76–123)	124 (78–156)	<0.001	<0.01	71	96 [21]	
Mn (μg/L)	5 (2–9)	6 (4–17)	6 (4–10)	0.3	0.7	1	1.0 [21]	12 [22]
Fe (μg/L)	0 (0–37)	797 (398–1292)	1075 (862–1324)	<0.001	<0.01	n/a	4690 [24]	476,000 [22]
Ni (μg/L)	12 (1–60)	5 (0–23)	26 (2–99)	0.3	0.7	n/a	2.2 [25]	2.7 [22]
Zn (μg/L)	179 (103–597)	1285 (678–1811)	537 (401–838)	<0.001	<0.05	283	474 [21]	5800 [22]
Cu (μg/L)	106 (55.3–196)	173 (128–257)	226 (130–314)	0.1	0.07	530	1189 [21]	830 [22]
Mo (μg/L)	0 (0)	0 (0–181)	0 (0)	<0.05	0.4	1	1.2 [21]	0.9 [22]
Pb (μg/L)	9 (4–21)	3 (1–6)	2 (1–5)	<0.05	<0.05	6	1.3 [21]	26 [25]

Values reported as median (interquartile range), [a]compared to normal subject by Mann–Whitney U test, data unavailable on [b]one patient, [c]three patients, [d]four patients, and [e]six patients, mg/L: milligrammes per litre, μL/L: microgrammes per litre, ng/mL: nanogrammes per millilitre, CF: Cystic fibrosis, NCFB: non-CF bronchiectasis.

Accurately characterising the presence of biometals will also provide information that will be relevant to the development of *in vitro* infection models and potentially, identify targets for novel therapies [11].

The strong positive correlation between the concentrations of Ca, Mg, Fe, Zn and Cu suggests that these metals may originate from a common disease process. LDH has previously been identified as a surrogate marker of pulmonary tissue damage and the positive correlation between several of the biometals detected and LDH levels supports cell injury as a probable contributor to the presence of some of these biometals [26]. The concentration of sputum biometals more closely represents levels seen in whole blood compared to serum (Table 1) and several metals (Mg, Fe and Zn) correlated positively with the concentration of pro-inflammatory IL-8, adding further support to the hypothesis that release of these metals is associated with local tissue damage.

Consistent with previous studies, CF subjects infected with *P. aeruginosa* had worse lung function and higher sputum IL-8 concentration, than their non-infected counterparts [27]. Surprisingly, there was a trend towards lower TCCs in the *P. aeruginosa* infected patients; however, sputum concentrations of Mg and Ca were significantly higher, which may again support a hypothesis that these metals are released from necrotic immune cells in the setting of heightened inflammation. From our results, it is not possible to determine whether the biometals originated from injured lung tissue or from luminal inflammatory cells and investigation of metal levels within the airway wall or lung parenchyma is required to address this question.

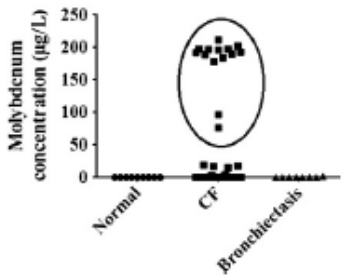


Fig. 1. Concentration of molybdenum in sputum samples.

Other potential sources of biometals in sputum should be considered, including micro-vascular leakage, cell membrane channelopathies and contamination from inhaled particulates. We have previously reported increased concentrations of microalbumin in CF sputa, which may suggest microvascular leakage is contributory [28], but, sputum biometal levels exceeded those reported in serum, which argues against this being the sole explanation [21–23].

CFTR-mutant airway epithelial cells (AECs) appear to "leak" Fe *in vitro*, which in-turn promotes growth of *P. aeruginosa* on their apical cell surface membrane, and suggests specific channelopathies may contribute to airway metal accumulation [29]. For example, divalent metal-ion transporter (DMT)-1 is important in airway Fe detoxification and, in DMT-1 deficient rats, Fe accumulates in the airways, resulting in increased oxidative stress and inflammation [30]. This observation is important, as abnormal function of metal transport channels may be amenable to therapeutic manipulation to limit the availability of metals to pathogenic bacteria.

The contribution of inhaled particulate matter to sputum metal concentrations was assessed by measurement of sputum of Pb and Ni concentrations and correlation of sputum biometal concentrations to atmospheric PM_{10} levels on the day of sample collection. Lead was detectable in the sputa of four CF subjects on admission to hospital, but not at subsequent time-points, raising the possibility that this metal was environmentally derived (e-Fig. 1). However, PM_{10} values did not correlate with sputum metal content in any of the subjects. Furthermore, Pb and Ni concentrations in subjects with CF and NCFB were not significantly different to those found

Table 3
Correlation co-efficients of biometals in all sputum samples[a,b,c].

	Ca	Mn	Fe	Zn	Cu	FEV_1 % predicted	TCC	IL-8	LDH	PM_{10}
Mg	0.89[d]	0.13	0.79[d]	0.75[d]	0.42[d]	−0.20	0.05	0.42[d]	0.44[d]	0.05
Ca	–	0.15	0.80[d]	0.67[d]	0.49[d]	−0.16	−0.02	0.23	0.33[d]	0.11
Mn	–	–	0.29[d]	0.12	0.06	0.28[e]	−0.13	0.15	−0.14	−0.14
Fe	–	–	–	0.75[d]	0.47[d]	−0.11	−0.08	0.39[d]	0.40[d]	0.03
Zn	–	–	–	–	0.46[d]	−0.18	0.02	0.38[d]	0.39[d]	0.03
Ni	–	–	–	–	–	−0.30[e]	−0.01	0.03	0.11	0.03
Cu	–	–	–	–	–	−0.20	−0.15	0.23	0.32[d]	0.04
Mo	–	–	–	–	–	−0.38[d]	−0.31	0.18	0.02	−0.03
Pb	–	–	–	–	–	−0.08	−0.13	−0.19	−0.10	−0.03

Values: Pearson's correlation-r values, [d]$p < 0.01$, [e]$p < 0.05$. [a]Correlations of Ni, Mo and Pb with other metals were not significant and are not included. [b]Correlations with FEV_1 % predicted, TCC, IL-8 and LDH do not include healthy control data. [c]Correlations with PM_{10} are based on 43 samples obtained from subjects with CF recruited from Queensland, where comparative air-quality data was available. FEV_1 % predicted: percentage predicted forced expiratory volume in one second. TCC: total inflammatory cell count. IL: interleukin. LDH: lactate dehydrogenase. PM_{10}: Particulate matter 10 μm or smaller in diameter.

in healthy controls. Taken together, these findings strongly suggest that environmental exposure to metals has minimal impact on sputum biometal content.

An important new finding is the identification of a distinct group of CF subjects with "elevated" concentrations of sputum Mo. Subjects with elevated Mo overall had more severe lung disease and a sub-group had CF related liver disease. Molybdenum is able to cycle between reduced (Mo^{4+}) and oxidised (Mo^{6+}) states under normal physiological conditions and facilitates the transfer of atomic oxygen between substrates in anoxic environments, which makes it essential to virtually all forms of life [16]. In humans, Mo co-factored proteins are incorporated into the flavoenzymes, which catalyse critical detoxification pathways [31]. Increased serum Mo concentrations have been reported in patients with liver disease, a finding not inconsistent with our sputum findings, although we did not assess Mo levels in serum to confirm a corresponding increase in systemic levels [32]. In bacteria, Mo is an essential component of nitrate reductase, which catalyses the denitrification of nitrate during anaerobic respiration [16]. Whilst *P. aeruginosa* is an aerobic bacterium, it can thrive under microaerobic and anaerobic conditions through the utilisation of nitrogen sources [33]. Under anaerobic conditions, *P. aeruginosa* increases the production of Mo containing proteins and mutant strains that are unable to incorporate Mo have attenuated virulence [34]. The evidence for anaerobic bacterial respiration in the diseased CF lung is compelling, with the presence of a variety of obligate anaerobes identified in addition to *P. aeruginosa* [33,35]. It is biologically plausible that airway Mo may promote anaerobic respiration in resident bacteria, but this requires further investigation. Interestingly, TCC was lower in the elevated Mo group, which as discussed previously, may suggest immune cell necrosis is contributing towards airway Mo. Longitudinal studies, which correlate Mo concentrations with, clinical status (stable *versus* pulmonary exacerbation), lung function decline, infecting pathogens, airway inflammation and serum Mo levels are required to further define the relevance to bacterial virulence and outcomes in patients with CF.

Increased Fe in the sputum of patients with CF has been demonstrated in several studies [9,28,36,37]. Iron poses a risk to the lung through generation of toxic hydroxyl radicals and oxidative stress *via* Fenton chemistry and, as previously discussed, Fe has been shown to influence *P. aeruginosa* behaviour *in vitro* [10]. Furthermore, Fe concentrations positively correlate with *P. aeruginosa* colony counts in the sputum of patients with CF confirming its importance *in vivo* [9].

Magnesium has generally been considered to be anti-inflammatory [38], but animal studies have shown that it may inhibit phagocytosis and impair the oxidative burst capacity of neutrophils, which will be detrimental in the setting of chronic infection [39]. From a bacterial perspective, conflicting *in vitro* studies suggest Mg may inhibit formation of new *P. aeruginosa* biofilms, but protect established biofilms from disruption [40].

Calcium was also present in high concentrations in sputa from patients with CF and NCFB, and *in vitro* studies have demonstrated that Ca-dependent signalling enhances *P. aeruginosa* cell adherence, including adherence to AECs [41]. Additionally, culture of *P. aeruginosa* in medium supplemented with 10 mM Ca^{2+} resulted in a 10 fold increase in biofilm thickness, and up-regulation of virulence factors, including a three-fold increase in production of pyocyanin [42]. Pyocyanin itself, which is an important *P. aeruginosa* exotoxin, disturbs AEC Ca homeostasis, which will serve to further increase airway Ca concentrations [43].

The high concentration of Zn found in CF and, to a lesser extent, NCFB sputa is consistent with a single, previous publication [8]. This finding is surprising given the high prevalence of systemic Zn deficiency reported in CF, and may therefore suggest selective Zn loss into the airways [18,44]. Human AEC strongly expresses the anti-oxidant Cu–Zn-SOD and systemic Zn deficiency increases airway inflammation and susceptibility to oxidative stress [14]. Zinc co-factored metalloproteinases, such as ADAM33, contribute to airway remodelling in response to repeated insults [45]. *In vitro* and animal models suggest Zn restores defective chloride transport by activating alternative Ca-dependent chloride channels and Zn supplementation has been shown to be of modest benefit in reducing antibiotic requirements in children with CF [44]. In bacteria, Zn is an essential metal, but also potentially toxic and its uptake is tightly regulated [12]. *P. aeruginosa* Zn uptake is regulated by the operon CzcRS, which in the presence of Zn promotes expression of the metal efflux pump CzcCBA. Additionally, CzcRS down regulates OprD that confers resistance to carbapenem antibiotics, suggesting an increase in Zn within the

airway may influence antibiotic susceptibility [12]. The net effect on disease outcomes in CF and NCFB of increased airway Zn concentrations remains uncertain, given the anti-inflammatory properties *versus* potential promotion of antibiotic resistance, and requires further investigation.

This study has a number of limitations. Studies of airway pathology based on expectorated sputum are intrinsically associated with sampling variability and confounded by the possibility of oropharyngeal contamination, but we were very careful to select only airway plugs and salivary contamination with this practice is minimal [46]. There were only a small number of healthy controls and patients with NCFB, but our numbers are comparable with earlier reports [8,36,37]. There was no change in sputum metal ion concentrations during treatment of a pulmonary exacerbation, suggesting that the measurement of metals has limited potential as a biomarker of therapeutic response. However, patients who provided serial samples were at the severe end of the disease spectrum. These individuals often respond sub-optimally to antibiotic treatment, in terms of their clinical improvement, reduction in bacterial numbers and inflammation, which may have limited our ability to detect meaningful changes [46]. Future studies should examine whether biometal concentrations fluctuate in response to treatment in patients with milder disease, and also at a much earlier time-point in the disease process, *i.e.* prior to the onset of infection. Additionally, studies in subjects at an earlier disease time-point may allow metal concentrations to be correlated with the presence of different inflammatory cell types (*i.e.* neutrophils *versus* macrophages), which is not possible in the adult subjects, with severe disease, where airway inflammation is dominated by neutrophils. Finally, analysis of the impact of specific bacterial infections, *e.g.* MRSA and *Burkholderia* species, on biometal levels is limited by the study size.

In summary, this is the most extensive analysis of sputum biometals in patients with CF and NCFB undertaken to date. The positive correlation between sputum LDH and biometal concentrations suggests that local cell damage is associated with airway metal content; however, whether local tissue injury is the source or a consequence of the presence of redox active biometals in the airway requires further investigation. A subgroup of CF patients with severe lung disease were identifiable by the presence of increased Mo in their sputum. It is likely that the complex composition of the biometals milieu in the lung strongly influences the behaviour of both the host immune response as well as bacterial pathogens. Mechanistic studies are required to establish the source of the metals detected and to determine their relevance to oxidative stress, impaired immune function and the promotion of bacterial virulence. Novel therapeutic interventions that modulate the availability of these biometals may reduce airway infection and substantially impact on disease progression.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jcf.2013.12.001.

Conflict of interest

Nil to declare.

Funding information

DJS, GJA and DWR are recipients of a Post-graduate Scholarship, Senior Research Fellowship and Practitioner Fellowship respectively from the National Health and Medical Research Council of Australia.

Acknowledgements

DJ Smith—Study design, patient recruitment, data analysis and manuscript preparation.

DW Reid—Guarantor of the study, study design, patient recruitment, data analysis and manuscript preparation.

SC Bell—Patient recruitment, manuscript review and editing.

GJ Anderson—Study design, manuscript review and editing.

References

[1] Flume PA, Mogayzel Jr PJ, Robinson KA, Goss CH, Rosenblatt RL, Kuhn RJ, et al. Cystic fibrosis pulmonary guidelines: treatment of pulmonary exacerbations. Am J Respir Crit Care Med 2009;180(9):802–8.

[2] Mott LS, Park J, Murray CP, Gangell CL, de Klerk NH, Robinson PJ, et al. Progression of early structural lung disease in young children with cystic fibrosis assessed using CT. Thorax 2012;67(6):509–16.

[3] Cystic Fibrosis Foundation patient registry (2010): annual data report. Maryland, USA: The Cystic Fibrosis Foundation, Bethesda; 2012.

[4] Pressler T. Targeting airway inflammation in cystic fibrosis in children: past, present, and future. Paediatr Drugs 2011;13(3):141–7.

[5] Sanderson K, Wescombe L, Kirov SM, Champion A, Reid DW. Bacterial cyanogenesis occurs in the cystic fibrosis lung. Eur Respir J 2008;32(2):329–33.

[6] Worlitzsch D, Tarran R, Ulrich M, Schwab U, Cekici A, Meyer KC, et al. Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. J Clin Invest 2002;109(3):317–25.

[7] Fraga CG. Relevance, essentiality and toxicity of trace elements in human health. Mol Aspects Med 2005;26(4–5):235–44.

[8] Gray RD, Duncan A, Noble D, Imrie M, O'Reilly DS, Innes JA, et al. Sputum trace metals are biomarkers of inflammatory and suppurative lung disease. Chest 2010;137(3):635–41.

[9] Reid DW, Carroll V, O'May C, Champion A, Kirov SM. Increased airway iron as a potential factor in the persistence of *Pseudomonas aeruginosa* infection in cystic fibrosis. Eur Respir J 2007;30(2):286–92.

[10] Glick R, Gilmour C, Tremblay J, Satanower S, Avidan O, Deziel E, et al. Increase in rhamnolipid synthesis under iron-limiting conditions influences surface motility and biofilm formation in *Pseudomonas aeruginosa*. J Bacteriol 2010;192(12):2973–80.

[11] Smith DJ, Lamont IL, Anderson GJ, Reid DW. Targeting iron uptake to control *Pseudomonas aeruginosa* infections in cystic fibrosis. Eur Respir J 2013;46(6):1723–36.

[12] Dieppois G, Ducret V, Caille O, Perron K. The transcriptional regulator CzcR modulates antibiotic resistance and quorum sensing in *Pseudomonas aeruginosa*. PLoS One 2012;7(5):e38148.

[13] Jomova K, Valko M. Advances in metal-induced oxidative stress and human disease. Toxicology 2011;283(2–3):65–87.

[14] Kinnula VL, Crapo JD. Superoxide dismutases in the lung and human lung diseases. Am J Respir Crit Care Med 2003;167(12):1600–19.

[15] MacNee W. Oxidative stress and lung inflammation in airways disease. Eur J Pharmacol 2001;429(1–3):195–207.

[16] Iobbi-Nivol C, Leimkuhler S. Molybdenum enzymes, their maturation and molybdenum cofactor biosynthesis in *Escherichia coli*. Biochim Biophys Acta 2013;1827(8–9):1086–101.

[17] Stojanovic D, Nikic D, Lazarevic K. The level of nickel in smoker's blood and urine. Cent Eur J Public Health 2004;12(4):187–9.

[18] Chen LC, Lippmann M. Effects of metals within ambient air particulate matter (PM) on human health. Inhal Toxicol 2009;21(1):1–31.

[19] Pizzichini E, Pizzichini MM, Efthimiadis A, Hargreave FE, Dolovich J. Measurement of inflammatory indices in induced sputum: effects of selection of sputum to minimize salivary contamination. Eur Respir J 1996;9(6):1174–80.
[20] Domej W, Krachler M, Schlagenhaufen C, Trinker M, Krejs GJ, Irsigolic KJ. Trace elements in pleural effusions. J Trace Elem Med Biol 1997;11(4):232–8.
[21] Rodushkin I, Odman F, Branth S. Multielement analysis of whole blood by high resolution inductively coupled plasma mass spectrometry. Fresenius J Anal Chem 1999;364(4):338–46.
[22] Minoia C, Sabbioni E, Apostoli P, Pietra R, Pozzoli L, Gallorini M, et al. Trace element reference values in tissues from inhabitants of the European community. I. A study of 46 elements in urine, blood and serum of Italian subjects. Sci Total Environ 1990;95:89–105.
[23] Prohaska C, Pomazal K, Steffan I. Determination of Ca, Mg, Fe, Cu, and Zn in blood fractions and whole blood of humans by ICP-OES. Fresenius J Anal Chem 2000;367(5):479–84.
[24] Goulle JP, Mahieu L, Castermant J, Neveu N, Bonneau L, Laine G, et al. Metal and metalloid multi-elementary ICP-MS validation in whole blood, plasma, urine and hair. Reference values. Forensic Sci Int 2005;153(1):39–44.
[25] Hood MI, Skaar EP. Nutritional immunity: transition metals at the pathogen–host interface. Nat Rev Microbiol 2012;10(8):525–37.
[26] Wark PA, Johnston SL, Moric I, Simpson JL, Hensley MJ, Gibson PG. Neutrophil degranulation and cell lysis is associated with clinical severity in virus-induced asthma. Eur Respir J 2002;19(1):68–75.
[27] Pukhalsky AL, Kapranov NI, Kalashnikova EA, Shmarina GV, Shabalova LA, Kokarovtseva SN, et al. Inflammatory markers in cystic fibrosis patients with lung *Pseudomonas aeruginosa* infection. Mediators Inflamm 1999;8(3):159–67.
[28] Reid DW, Lam QT, Schneider H, Walters EH. Airway iron and iron-regulatory cytokines in cystic fibrosis. Eur Respir J 2004;24(2):286–91.
[29] Moreau-Marquis S, Bomberger JM, Anderson GG, Swiatecka-Urban A, Ye S, O'Toole GA, et al. The DeltaF508-CFTR mutation results in increased biofilm formation by *Pseudomonas aeruginosa* by increasing iron availability. Am J Physiol Lung Cell Mol Physiol 2008;295(1):L25–37.
[30] Kim J, Molina RM, Donaghey TC, Buckett PD, Brain JD, Wessling-Resnick M. Influence of DMT1 and iron status on inflammatory responses in the lung. Am J Physiol Lung Cell Mol Physiol 2011;300(4):L659–65.
[31] Sardesai VM. Molybdenum: an essential trace element. Nutr Clin Pract 1993;8(6):277–81.
[32] Yoshida M, Ota S, Fukunaga K, Nishiyama T. Serum molybdenum concentration in healthy Japanese adults determined by inductively coupled plasma-mass spectrometry. J Trace Elem Med Biol 2006;20(1):19–23.
[33] Schobert M, Jahn D. Anaerobic physiology of *Pseudomonas aeruginosa* in the cystic fibrosis lung. Int J Med Microbiol 2010;300(8):549–56.
[34] Noriega C, Hassett DJ, Rowe JJ. The mobA gene is required for assimilatory and respiratory nitrate reduction but not xanthine dehydrogenase activity in *Pseudomonas aeruginosa*. Curr Microbiol 2005;51(6):419–24.
[35] Tunney MM, Field TR, Moriarty TF, Patrick S, Doering G, Muhlebach MS, et al. Detection of anaerobic bacteria in high numbers in sputum from patients with cystic fibrosis. Am J Respir Crit Care Med 2008;177(9):995–1001.
[36] Stites SW, Plautz MW, Bailey K, O'Brien-Ladner AR, Wesselius LJ. Increased concentrations of iron and isoferritins in the lower respiratory tract of patients with stable cystic fibrosis. Am J Respir Crit Care Med 1999;160(3):796–801.
[37] Stites SW, Walters B, O'Brien-Ladner AR, Bailey K, Wesselius LJ. Increased iron and ferritin content of sputum from patients with cystic fibrosis or chronic bronchitis. Chest 1998;114(3):814–9.
[38] Tam M, Gomez S, Gonzalez-Gross M, Marcos A. Possible roles of magnesium on the immune system. Eur J Clin Nutr 2003;57(10):1193–7.
[39] Bussiere FI, Gueux E, Rock E, Girardeau JP, Tridon A, Mazur A, et al. Increased phagocytosis and production of reactive oxygen species by neutrophils during magnesium deficiency in rats and inhibition by high magnesium concentration. Br J Nutr 2002;87(2):107–13.
[40] Mulcahy H, Lewenza S. Magnesium limitation is an environmental trigger of the *Pseudomonas aeruginosa* biofilm lifestyle. PLoS One 2011;6(8):e23307.
[41] Johnson MD, Garrett CK, Bond JE, Coggan KA, Wolfgang MC, Redinbo MR. *Pseudomonas aeruginosa* PilY1 binds integrin in an RGD- and calcium-dependent manner. PLoS One 2011;6(12):e29629.
[42] Sarkisova S, Patrauchan MA, Berglund D, Nivens DE, Franklin MJ. Calcium-induced virulence factors associated with the extracellular matrix of mucoid *Pseudomonas aeruginosa* biofilms. J Bacteriol 2005;187(13):4327–37.
[43] Denning GM, Railsback MA, Rasmussen GT, Cox CD, Britigan BE. *Pseudomonas* pyocyanine alters calcium signaling in human airway epithelial cells. Am J Physiol 1998;274(6 Pt 1):L893–900.
[44] Abdulhamid I, Beck FW, Millard S, Chen X, Prasad A. Effect of zinc supplementation on respiratory tract infections in children with cystic fibrosis. Pediatr Pulmonol 2008;43(3):281–7.
[45] Holgate ST, Yang Y, Haitchi HM, Powell RM, Holloway JW, Yoshisue H, et al. The genetics of asthma: ADAM33 as an example of a susceptibility gene. Proc Am Thorac Soc 2006;3(5):440–3.
[46] Sagel SD, Chmiel JF, Konstan MW. Sputum biomarkers of inflammation in cystic fibrosis lung disease. Proc Am Thorac Soc 2007;4(4):406–17.

Appendix 2: Appendix to Chapter 3

The following appendix constitutes data mentioned in Chapter 3: Response of the CF lung Microbiome to intravenous antibiotics.

Appendix 2.1: Intravenous antibiotic usage, standard microbiological cultures and sensitivity testing results for patients enrolled in the study which constitutes Chapter 3

Appendix 2.2: Demographics, lung function and antibiotic treatment of individual patients enrolled in the study which constitutes Chapter 3

Appendix 2.3: Taxonomic classification of 16s rDNA reads from sputum samples analysed in the study which constitutes Chapters 3

Appendix 2.1: Intravenous antibiotic usage, standard microbiological cultures and sensitivity testing results for patients enrolled in the study which constitutes Chapter 3

			Antibiotic sensitivity testing															
Subject	Intravenous antibiotics	Standard microbiological culture results	Gentamicin	Tobramycin	Amikacin	Ceftazadime	Timentin	Piperacillin	Tazocin	Aztreonam	Meropenem	Imipenem	Ciprofloxacin	Colisitin	Bactrim	Minocycline	Flucloxacillin	Vancomycin
P1	Ceftazadime, Tobramycin, Azithromycin	Mucoid Psa	R	S	R	S	S	S		S	S		S	S				
		Non-Mucoid Psa	R	S	R	R	R	S		R	R		R	S				
P2	Ceftazadime, Tobramycin, Azithromycin	Mucoid Psa	R	R	S	S	S	S		S	S		S	S				
		Non-Mucoid Psa	S	S	S	S	S	S		S	S		S	S				
		S. Maltophilia													R	S		
P3	Meropenem, Tobramycin, Azithromycin	Mucoid Psa	R	R	R	R	R	R		R	R		R	S				
P4	Meropenem, Ceftazadime, Tobramycin, Azithromycin	Mucoid Psa	S	S	S	S	S	S		S	S		S	S				
		Non-Mucoid Psa	R	R	R	R	R	R		R	R		R	S				
		MSSA															S	
P5	Meropenem, Ceftazadime, Tobramycin, Azithromycin	Mucoid Psa	R	S	R	S	S	S		S	S		S	S				
		Non-mucoid Psa	R	R	R	S	R	R		S	S		S	S				
P6	Ceftazadime, Tobramycin, Azithromycin	Mucoid Psa	R	S	R	R	R	R		R	S		S	S				
		Non-mucoid Psa	R	R	R	R	R	R		R	S		R	S				
		A. fumigatus																
P7	Ceftazadime, Tobramycin, Azithromycin	Mucoid Psa	S	S	S	S	S	S		S	S		S	S				
		Non-mucoid Psa	R	R	R	S	R	S		R	S		R	S				

P8	Meropenem, Ceftazadime, Tobramycin, Azithromycin	Mucoid Psa	R	R	R	R	R	R		R	R		R	S				
		Non-mucoid Psa	R	S	R	R	R	S		S	R		S	S				
P9	Ceftazadime, Tobramycin, Azithromycin	Mucoid Psa	R	S	R	S	S	S		S	S		S	S				
		Non-mucoid Psa	R	S	R	S	S	S		S	R		S	S				
P10	Ceftazadime, Tobramycin, Azithromycin	Mucoid Psa	S	S	S	R	R	R		R	S		S	S				
		MRSA															R	S
P11	Meropenem, Aztreonam, Tobraymicn	Mucoid Psa	R	S	R	S	S	S		S	S		S	S				
		Non-mucoid Psa	R	R	R	R	R	R		R	R		R	R				
P12	Ceftazadime Tobramycin	Mucoid Psa	R	R	R	S	S	S		S	S		S	S				
		Non-Mucoid Psa	R	S	R	R	R	R		R	R		S	S				
		MSSA															S	
P13	Ceftazadime, Tobramycin	Mucoid Psa	S	S	S	S	S	S		S	S		S	S				
		Non-mucoid Psa	R	R	R	S	S	S		S	S		S	S				
P14	Colistin, Meropenem, Ceftazadime	Mucoid Psa	R	R	R	R	R	R		R	R		R	S				
		Non-mucoid Psa	R	R	R	R	R	R		R	S		S	S				
P15	Colistin, Meropenem, Ceftazadime	Mucoid Psa	R	S	R	R	R	R		S	S		S	S				
		A. fumigatus																
P16	Ceftazadime, Tobramycin, Azithromycin	Mucoid Psa	R	S	R	S	S		S	S		S	S					
		C. albicans																
		MRSA																
P17	Meropenem, Inhaled Tobramycin	Mucoid Psa	R	S	R	S	S		S	S		S	R	S				
		Non-mucoid Psa	R	R	R	R			R	R		R	R	S				
P18	Ceftazadime, Tobramycin,	Mucoid Psa	S			S	S		S	S			S					
		Non-mucoid Psa	S			S	S		S	S			S					

	Azithromycin	MSSA																
		A. fumigatus																
	Timentin, Tobramycin, Azithromycin	Mucoid Psa	R	S	S	S	S		S	S		S	S					
		Non-mucoid Psa	R	R	R	S	S		S	S		S	R					
P19		*Penicillium Spp*.																
	n/a	Mucoid Psa	S			S	S			S			R					
		MSSA																
P20		*S. prolificans*																
	Tazocin, Tobrmycin, Azithromycin, Bactrim	Mucoid Psa	R	R	R	R	R		S	S		R	R					
		C. albicans																
P21		*A. fumigaus*																
	Ceftazadime, Tobramycin, Azithromycin	Mucoid Psa	S	S	S	S	S		S	S		S	S					
		Mucoid Psa	R	R	R	S	S		S	S		S	S					
P22		*C. albicans*																
	Ceftazadime, Tobramycin, Azithromycin	Mucoid Psa	S	S	R	S	S		S	S		S	R					
P23		*C. albicans*																
	Ceftazadime, Tobramycin, Azithromycin	Mucoid Psa	S			S	S		S	S			R					
		Non-mucoid Psa	R			S	S		S	S			R					
P25		MSSA																

Psa: *Pseudomonas aeruginosa*, *S. maltophilia*: *Stenotrophomonas maltophilia*, MSSA: methicillin sensitive *Staphylococcus aureus*, MRSA: methicillin resistant *Staphylococcus aureus*, A. fumigatus: *Aspergillus fumigatus*, *C. albicans*: *Candida albicans*, *S. prolificans*: *Scedosporium prolificans*. S: sensitive, R: resistant, Px: subject number.

Appendix 2.2: Demographics, lung function and antibiotic treatment of individual patients enrolled in the study which constitutes Chapter 3

Patient Code	Sex	Age (years)	Treatment Location	Genotype	BMI	Best FEV1 in the Previous 6 months (L)	Best FEV1 % Predicted in the previous six	FEV1 Prior to treatment (L)	FEV1 Percent predicted prior to treatment	FEV1 Post Treatment (L)	FEV1 Percent predicted post treatment	Relative improvement (%)	Absolute Improvement (%)	FEV1 severity*
P1	M	18	Queensland	DF508Homo	18.7	1.29	33%	0.93	24%	0.97	25%	4%	1%	Severe
P2	M	32	Queensland	DF508/Unknown	23.9	1.24	29%	1.07	25%	1.24	29%	16%	4%	Severe
P3	M	18	Queensland	DF508Homo	19.3	1.21	30%	1.13	26%	1.21	28%	8%	2%	Severe
P4	M	29	Queensland	DF508Homo	20.6	0.87	21%	0.87	21%	n/a	n/a	n/a	n/a	Severe
P5	M	27	Queensland	DF508Homo	25.7	3.37	84%	n/a	n/a	3.21	80%	n/a	n/a	Mild
P6	F	34	Queensland	DF508Homo	20.8	1.60	50%	1.20	38%	1.44	45%	18%	7%	Moderate
P7	F	29	Queensland	DF508/N1303k	17.9	1.71	60%	1.23	44%	1.53	55%	25%	11%	Moderate
P8	M	22	Queensland	DF508/Unknown	17.1	1.99	47%	1.39	33%	1.87	45%	36%	12%	Moderate
P9	M	21	Queensland	DF508/Unknown	18.4	1.40	37%	1.18	31%	1.36	36%	16%	5%	Severe
P10	M	37	Queensland	DF508Homo	24.5	1.21	30%	0.79	20%	0.83	22%	10%	2%	Severe
P11	M	31	Queensland	DF508/Unknown	21.9	1.50	28%	1.13	28%	1.33	33%	18%	5%	Severe
P12[#]	M	27	Queensland	DF508Homo	26.0	4.17	97%	3.73	87%	4.17	97%	10%	10%	Mild
P13[#]	M	27	Queensland	DF508Homo	25.1	4.19	97%	3.84	90%	4.16	96%	7%	6%	Mild
P14	F	20	Queensland	G551/R1162X	16.8	0.99	29%	0.76	22%	0.99	28%	29%	6%	Severe
P15	F	52	Queensland	DF508/Unknown	19.8	0.91	35%	0.89	34%	0.91	35%	2%	1%	Severe
P16	F	25	Tasmania	DF508Homo	18.7	1.14	36%	0.85	27%	1.13	36%	33%	9%	Severe
P17	F	29	Tasmania	DF508/1898+1G	18.5	2.24	68%	1.77	54%	1.81	56%	4%	2%	Moderate
P18	M	22	Tasmania	DF508Homo	20.2	2.45	58%	1.59	38%	2.79	67%	76%	29%	Moderate

P19	M	26	Tasmania	DF508Homo	21.8	2.86	67%	2.85	67%	2.87	68%	1.5%	1%	Moderate
P20	M	19	Tasmania	DF508/Unknown	20.7	3.27	75%	2.32	53%	3.40	76%	43%	23%	Mild
P21	M	54	Tasmania	G542x/Unknown	20.7	2.14	55%	1.71	43%	1.67	42%	0%	0%	Moderate
P22	M	35	Tasmania	DF508Homo	19.8	1.95	55%	1.50	37%	1.87	46%	24%	9%	Moderate
P23	M	19	Tasmania	R553X/1717-1G>A	17.9	2.06	51%	n/a	n/a	2.00	50%	n/a	n/a	Moderate
P24	M	18	Tasmania	DF508Homo	19.8	3.89	93%	3.79	91%	4.02	94%	3.3%	3%	Mild

F: female, M: male, BMI: body mass index, L: litres, *Based on best lung function values obtained in the six months prior to treatment, FEV_1 >70% mild, 40-70% moderate, <40% severe impairment. # Separate exacerbation series collected from the same patient, 12 months apart. n/a: not available

Appendix 2.3: Taxonomic classification of 16s rDNA reads from sputum samples analysed in the study which constitutes Chapters 3

Kingdom	TP-1 [%]	TP-2 [%]	TP-3 [%]
Bacteria	100	100	100
Phyllum			
Proteobacteria	90.65	56.95	84.7
Firmicutes	4.25	17.7	6.2
Bacteroidetes	1.5	8.4	0.3
Actinobacteria	0.25	1.4	0.2
Fusobacteria	0.15	0.7	0
Class			
Gammaproteobacteria	90.55	56.45	84.7
Bacilli	2.85	9.05	5.5
Bacteroidia	1.5	7.9	0.3
Clostridia	1.05	2.75	0.7
Actinobacteria	0.25	1.4	0.2
Fusobacteria	0.15	0.7	0
Betaproteobacteria	0	0.05	0
Order			
Pseudomonadales	90.3	38.45	74.6
Lactobacillales	2.85	8.65	5.5
Bacteroidales	1.5	7.9	0.3
Clostridiales	1.05	2.75	0.7
Actinomycetales	0.2	0.55	0.2
Fusobacteriales	0.15	0.7	0
Gammaproteobacteria_incertae_sedis	0.1	0.05	0.1
Burkholderiales	0	0.05	0
Coriobacteriales	0	0.05	0
Unclassified	0.25	0.25	0.4

Family			
Pseudomonadaceae	90.3	38.4	74.6
Streptococcaceae	2.7	7.4	5.5
Prevotellaceae	1.25	7.6	0.3
Veillonellaceae	1.05	2.75	0.7
Fusobacteriaceae	0.15	0	0
Micrococcaceae	0.1	0.5	0.1
Simiduia	0.1	0.05	0.1
Carnobacteriaceae	0	0.05	0
Coriobacteriaceae	0	0.05	0
Unclassified	0.35	0.65	0.4
Genus			
Pseudomonas	69.5	23.45	58.6
Streptococcus	2.7	7.4	4
Prevotella	1.25	6.9	0.3
Veillonella	0.75	2.65	0.7
Azomonas	0.3	0.1	0.2
Fusobacterium	0.15	0	0
Rothia	0.1	0.5	0.1
Atopobium	0	0.05	0
Granulicatella	0	0.05	0
Unclassified	12.9	6.45	13.8

Appendix 3: Appendix to Chapter 4

The following appendix constitutes data mentioned in Chapter 3: Response of the CF lung Microbiome to intravenous antibiotics.

Appendix 3.1. Sputum microbiology of CF patients enrolled in the study which constitutes Chapter 3

Subject No.	Gender	Age	FEV1 percentage predicted	Body Mass Index	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	Methicillin resistant *Staphylococcus aureus*	*Achromobacter xylosoxidans*	*Burkholderia gladioli*	*Scedosporium apiospermum*	*Chryseobacterium indologenes*	*Stenotrophomonas maltophilia*	*Aspergillus fumigatus*	*Haemophilus influenae*	*Mycobacterium avian intracellulare*
1	Male	29	24.0	19.4	✓										
2	Female	19	73.4	20.0	✓										
3	Male	28	82.1	27.7	✓										
4	Male	27	56.2	24.2	✓								✓		
5	Male	32	27.4	24.1	✓							✓			
6	Male	22	58.0	19.5	✓	✓									
7	Female	32	65.6	23.4	✓	✓								✓	
8	Female	27	62.9	20.5	✓						✓				
9	Female	21	67.7	23.5	✓					✓					
10	Male	35	52.5	24.8	✓	✓		✓							
11	Male	31	21.5	24.5	✓		✓								
12	Male	25	56.7	37.3	✓									✓	
13	Female	34	69.7	27.3	✓	✓									
14	Male	26	83.6	22.4	✓										
15	Male	28	100.1	24.9	✓	✓							✓		
16	Male	26	51.0	23.3	✓	✓									
17	Female	22	79.5	21.0	✓										
18	Male	28	27.6	20.5	✓						✓				
19	Male	24	41.6	26.5	✓										
20	Male	40	23.6	25.4	✓										
21	Female	22	78.6	22.6	✓										
22	Female	33	69.5	18.5	✓										
23	Male	32	80.3	27.8	✓										
24	Female	34	64.3	27.1	✓										
25	Male	38	32.6	20.0	✓	✓									

26	Female	20	94.2	26.3	✓	✓									
27	Female	27	53.3	19.1	✓										
28	Male	31	28.0	23.8	✓										
29	Female	25	76.2	24.3	✓	✓									
20	Male	36	53.9	26.2	✓										
31	Male	20	60.7	23.0	✓								✓		
32	Male	34	31.5	24.0	✓	✓						✓			
33	Male	27	34.1	21.5	✓										
34	Male	30	48.0	21.2	✓										
35	Female	24	39.6	19.2	✓										
36	Female	17	32.6	17.8								✓	✓		
37	Male	43	66.9	24.7					✓						
38	Female	18	50.3	17.7						✓					✓
39	Female	18	99.0	31.0									✓	✓	
40	Male	23	78.7	22.1		✓								✓	
41	Female	40	67.8	32.4		✓									